MEDICAL LIBRARY
MAY 15 1950

THIS PART COMPLETES VOLUME 45 (1949)

1949 VOLUME 45, No. 5

THE BIOCHEMICAL JOURNAL

Edited for

THE BIOCHEMICAL SOCIETY

Editorial Board

R. K. CALLOW
G. D. GREVILLE
R. A. KEKWICK
E. J. KING (*Chairman*)
W. KLYNE
M. G. MACFARLANE
H. McILWAIN
R. A. MORTON
A. NEUBERGER
A. PIRIE
R. L. M. SYNGE
R. H. S. THOMPSON

CAMBRIDGE UNIVERSITY PRESS
CAMBRIDGE, AND BENTLEY HOUSE, LONDON
AGENTS FOR U.S.A.
THE UNIVERSITY OF CHICAGO PRESS
CANADA, INDIA, AND PAKISTAN: MACMILLAN

Price 20s. net

The *Biochemical Journal* is conducted by the **Biochemical Society** and is published by the Cambridge University Press. The *Journal* is issued in multiple volumes per year. Each volume consists of approximately 640 pages, published in five parts at intervals of one month.

The Biochemical Society was instituted solely for the advancement of the Science of Biochemistry, and holds meetings at different centres for the communication of original papers, and for the discussion of current problems. Persons interested in Biochemistry are eligible for election. Members whose subscriptions are not in arrears are entitled to receive the *Biochemical Journal* without further payment. Further information may be obtained from the Hon. Secs., Professor J. N. Davidson, Department of Biochemistry, The University, Glasgow, W. 2, and Dr J. H. Birkinshaw, London School of Hygiene and Tropical Medicine, Keppel Street, London, W.C. 1, or from the Hon. Treas., Dr J. H. Bushill, 149 Hammersmith Road, London, W. 14.

Subscriptions to the *Biochemical Journal*. For non-members of the Biochemical Society the subscription to the *Biochemical Journal* is £3. 10*s*. 0*d*. per volume, payable in advance to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1, to Dr J. H. Bushill, The Laboratories, 149 Hammersmith Road, London, W. 14, or to any bookseller. In the United States of America the *Journal* may be purchased from the University of Chicago Press, 5750 Ellis Avenue, Chicago, Ill., the American agents of the Cambridge University Press for the sale of the *Biochemical Journal*.

Communications respecting the sale of single issues or back numbers of the *Journal* should be addressed to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1.

Claims for the replacement of *Journals* lost in transmission will not be entertained if they are received later than three months after the date of the posting of the *Journal*.

Prices of back numbers of the *Journal*.

	In paper covers
Volume 17 complete.	£3 net
Volume 18, No. 2.	15*s*. net
Volume 19, No. 6.	£1 net
Volume 20 to 25 inclusive.	£3 net each
Volume 26 to 28 inclusive.	£3. 10*s*. net each
Volume 29, Nos. 1, 2 and 4 to 12 inclusive.	7*s*. 6*d*. net each
Volume 30, Nos. 4 to 8, 10 and 11.	7*s*. 6*d*. net each
Volume 30, Index.	2*s*. 6*d*. net
Volume 31, Nos. 1, 2, 3, 5 to 8, and 11.	7*s*. 6*d*. net each
Volume 31, Index.	2*s*. 6*d*. net
Volume 32, No. 8.	7*s*. 6*d*. net
Volume 32, Index.	2*s*. 6*d*. net
Volume 33, Nos. 8 to 12 inclusive.	7*s*. 6*d*. net each
Volume 33, Index.	2*s*. 6*d*. net
Volume 34, 35 and 36.	£3. 10*s*. net each
Volume 38, Reprinting.	
Volume 40 and 41.	£3. 10*s*. net each
Volume 42, Reprinting.	
Volume 43 and 44.	£3. 10*s*. net each
Index to Volumes 1–10.	5*s*. 3*d*. net
Index to Volumes 11–20.	10*s*. net
Index to Volumes 21–30.	15*s*. net
Index to Volumes 31–40.	£1. 15*s*. net

Binding. Quotations can be given by the publishers for bound copies of back numbers; also for buckram binding cases, and for binding subscribers' sets. (Suspended.)

The Potato Eelworm Hatching Factor

1. THE PREPARATION OF CONCENTRATES OF THE HATCHING FACTOR AND A METHOD OF BIOASSAY

BY C. T. CALAM, H. RAISTRICK AND A. R. TODD*
Biochemistry Department, London School of Hygiene and Tropical Medicine, and Chemistry Department, University of Manchester

(*Received* 8 *April* 1949)

The potato eelworm, *Heterodera rostochiensis* Wollenweber (Figs. 1 and 4), is a strain or subspecies of the small nematode, *H. schachtii*, and until comparatively recently was generally described under the latter name. Eelworms parasitize a variety of economically important crops and are a serious menace to agriculture in many parts of the world. The parasite was first described by Schacht (1859); it was named *H. schachtii* by Schmidt (1871), who recognized it as a prevalent pest on sugar beet in Germany. In that country, after 1836 when sugar beet first became a farm crop, steadily increasing production without any regard to rotation led to a vast increase in eelworm infestation, with the result that between 1870 and 1880 crop failures became frequent. Only by suitable rotational cropping and intensive cultivation could the pest be brought under control. In England, Massee (1913) described the disease known as 'potato sickness', the symptoms being slow growth, spindly stems and unhealthy foliage, and noted that it seemed to be associated with the presence of eelworms in the soil. Increases in the disease and in eelworm population were rapid in the period of intensive potato cultivation during the 1914–18 war, and potato sickness has now become, in this country, one of the most serious and widespread of potato diseases, having become even more prevalent during the recent world war. It should, perhaps, be mentioned that the severity of potato sickness may also be affected by unknown factors, as it cannot always be directly correlated with the population of potato eelworm cysts (cf. Buckhurst & Fryer, 1930; Ellenby, 1942).

Although *H. schachtii* had been reported as attacking, amongst other plants, beet, potatoes, oats and peas, it was observed that each strain became specialized to a single host and lost its ability to parasitize others. With this specialization came small changes in morphology, so that a subdivision of *H. schachtii* became necessary. The potato strain, characterized by the almost spherical shape of the mature cysts (Fig. 2) as distinct from the lemon-shaped cysts of the beet strain, was given the name *H. rostochiensis* by Wollenweber (1923).

The life history of *H. rostochiensis* is well known. The larvae hatched from cysts in the soil enter the growing potato plant after some 17 days by drilling a hole through the root wall. There the worms are true parasites until they attain maturity, when the males escape into the soil and the females remain attached to the roots by the head. The body of the fertilized female swells to a white roundish cyst full of undeveloped eggs, gradually becomes brown and then contains several hundred fully formed larvae (Figs. 5 and 6), and drops off into the surrounding soil as a mature cyst. The cysts lie dormant in the soil until stimulated to hatch by the growing of a new crop of potatoes. They can remain dormant but viable for several years, and indeed cases have been reported where cysts were still in part viable after 10 years. The disastrous effect of continued cropping on the same soil is thus readily understood, as is the value of a rotational system with several years between successive potato crops.

The hatching of larvae from cysts of *H. rostochiensis* occurs in response to a specific stimulus due to a secretion from the roots of the host (Fig. 7). This appears to have been first realized by Baunacke (1922); in the absence of the stimulus the cysts remain dormant, and hardly any larvae hatch (Fig. 3). Baunacke tried the effect of various chemical substances as hatching agents and claimed that ferric chloride, potassium permanganate and bleaching powder had some effect. O'Brien & Prentice (1930) showed that potato eelworm cysts could be made to hatch with 'potato root excretion' but not with secretions from beet, rape, mustard or oats. Triffitt (1930) was the first worker to investigate 'potato root excretion'. This author showed that it was non-volatile and reasonably heat resistant, and that it retained its activity for some time in sterile water. Later, Hurst (1935, 1937) attempted to isolate the hatching factor from leachings of soil in which potatoes were growing. By evaporation and ethanol precipitation he obtained powders which brought about hatching at a limiting dilution of

* Present address: University Chemical Laboratory, Pembroke Street, Cambridge.

1/500,000. On very scanty evidence he formed the opinion that the factor was an amino-acid and stated that several amino-acids, in particular tyrosine, showed some activity.

The isolation and structural elucidation of the potato eelworm hatching factor are problems of considerable scientific interest, but might also have very important practical consequences for agriculture. Much work has been devoted to the control of the pest, but no really satisfactory means of eradication has been found. Various chemicals have been used to treat the soil; of these, calcium cyanamide and chloroacetate seem to have shown some promise, but are uneconomic. More recently, dichloropropene-dichloropropane mixtures have been used to control, if not to eradicate, eelworm; such mixtures have been recommended in this country for the control of *H. marioni* in greenhouse plants (cf. Ministry of Agriculture and Fisheries, 1947). Recently, Ellenby (1945) has reported satisfactory results in experiments in which allyl *iso*thiocyanate is applied to soil infested with eelworm; these findings, which parallel the earlier observations of Smedley (1939) with phenyl *iso*thiocyanate, may explain the low incidence of eelworm attack on potatoes grown together with white mustard on infested soil (Morgan, 1925). The main difficulty lies, of course, in the dormancy of the cysts, for until the larvae emerge they are largely protected from attack by many chemical agents. This being so, an obvious alternative would be to apply a hatching factor to infected soil in the absence of potato or other plants capable of being parasitized by the eelworms; the emergent larvae could be allowed to die in the absence of a suitable host, or alternatively, would be readily open to attack by chemical agents. The natural hatching factor might well prove too complex for economic synthesis on the large scale needed if it were to be so used, but if its structure were known it might be hoped that some simpler related substance might have a similar action and be more easily prepared.

The investigations described in the present paper were initiated in 1939 at the London School of Hygiene and Tropical Medicine and at Winches Farm, St Albans, where the production of material was first undertaken with the help of Miss M. Oliver; they were transferred to the University of Manchester at the beginning of 1941 and continued there. At the outset it was clear that the methods employed by Hurst (1935, 1937) on potato root excretion were too cumbrous. Following a suggestion made by Prof. R. T. Leiper, whose assistance in the early stages of the work is gratefully acknowledged, it was decided to use the leachings from growing tomato plants instead of potatoes, since he had found that such leachings were highly active in hatching potato eelworms; moreover, tomatoes have the added advantage that they are more conveniently grown and handled in bulk indoors. The method finally adopted for preparing crude solid concentrates of the hatching factor is described in detail below. Plants were grown in soil in pots or trays and watered frequently, the leachings being collected. From the leachings, active material was adsorbed on activated charcoal from which it was eluted with aqueous acetone; evaporation of the eluate under reduced pressure gave a brownish solid showing high activity when tested on potato eelworm cysts. The amounts of material obtained were not very large. For example, continuous cultivation of 4000–5000 plants yielded in the course of 1 year some 27 g. of the crude solid mentioned above; this solid had an average Relative Activity (R.A.) of 6·5 (see p. 518) and probably contained less than 2 % of the factor itself.

EXPERIMENTAL

Isolation of crude material containing hatching factor

The preparation of hatching factor concentrates can be conveniently divided into four sections: (1) leaching, (2) adsorption, (3) elution and (4) evaporation.

(1) *Growing and leaching of tomato plants.* As production of fruit was not required the plants were kept quite small; constant over watering had in any case a bad effect on their development. Such experiments as were carried out showed that the most active leachings were obtained when the plants were 6–9 in. high with vigorously growing roots. It was thus necessary to keep a good supply of new plants coming along and to discard them after about 3 months, since by that time the yield of hatching factor had fallen off very markedly. Old plants could be 'reconditioned' by cutting off the tops and replanting the roots in fresh soil, but on the whole it was found better to discard them and use new seedlings.

The plants were established under the usual conditions. The seedlings were planted in 3 in. pots containing a suitable tomato-growing compost. After about 6 weeks they were well developed and leaching was begun. To facilitate collection of the leachings the pots were kept on tables with sloping tops made of asbestos sheeting or of boards covered with rubber 'roofing felt'. The tables were usually about 8 ft. long with a slope of 3 in. to one end, and some 4 ft. 6 in. wide with a slope of 3 in. towards a central gutter to carry off the leachings to an enamelled container. A table of this size held 215 plants. Watering was carried out on alternate days using water at the same temperature as the greenhouse. The space of about 1 in. between the earth and the top of each pot was usually filled with water which was allowed to soak in and the process repeated once. From 4000–5000 plants treated in this way, the yield of leachings was about 100 l./day, giving about 500 mg. of crude active solid when worked up as described below.

(2) *Adsorption.* The leachings, which were light orange in colour and had pH about 6·5, were poured without filtration into vessels containing charcoal (British Drug Houses Ltd., 'Activated Charcoal for Decolorizing Purposes' was found most suitable), 1 g. charcoal being used/l. of leachings. The charcoal gradually settled out on standing, and after 1 day the supernatant liquid was siphoned off and discarded. The charcoal adsorbate was collected on a Buchner funnel and

Fig. 1. *Heterodera rostochiensis*. The potato eelworm. Magnification, ×130.

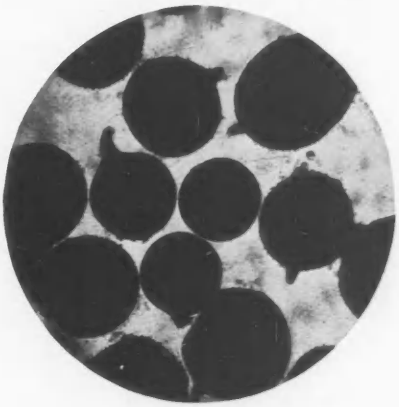


Fig. 2. Cysts of the potato eelworm. Magnification, ×30.

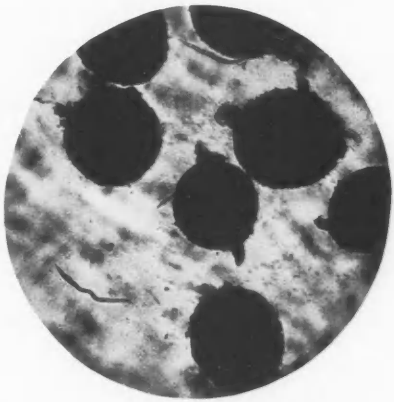


Fig. 3. Cysts of the potato eelworm soaking in water; two worms have hatched. Magnification, ×30.

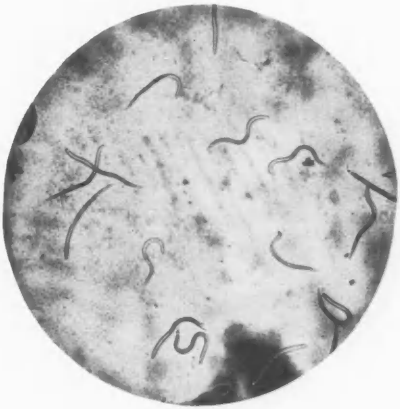


Fig. 4. Potato eelworms hatched from cysts. Magnification, ×30.

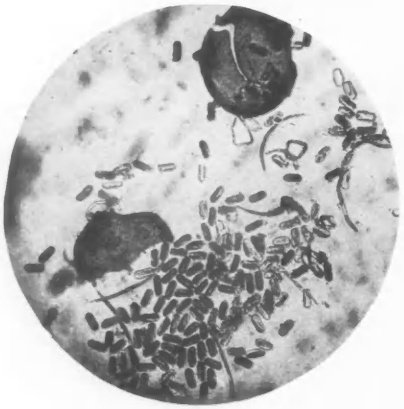


Fig. 5. A dissected cyst of the potato eelworm; note the immature larvae and a few mature worms. Magnification, ×30.

Fig. 6. Immature larvae and part of a mature eelworm. The typical coiled form of the worm in the larva can be discerned. Magnification, ×130.

Fig. 7. Cysts of the potato eelworm soaking in a solution of hatching factor. Twenty worms have hatched (cf. Fig. 3). Magnification, ×30.

sucked as dry as possible. The loss of activity in this process was probably about 10%. Various other adsorbents were tried and found inferior to charcoal.

(3) *Elution.* The moist charcoal adsorbate was generally collected and eluted in 1·5 kg. batches by stirring with 8 l. of aqueous acetone (70% acetone) in four lots. The eluate was pale yellow; the charcoal recovered could not be used again and was discarded.

(4) *Evaporation.* The filtered eluate was evaporated under a pressure of 15 mm., the temperature being kept below 30° and the evaporation residue finally dried in a desiccator at room temperature over H_2SO_4. The product was a light brown powder, readily soluble in water, and contained some 60–70% of the active material in the leachings. It formed the starting material for all the investigations here described. During the year 1940, before the concentration procedure had been standardized, a total of 76 g. crude solid of average R.A. 1 was obtained from plants grown at Winches Farm, St Albans. During 1941, 27 g. solid of average R.A. 6·5 was obtained from the same source, together with 9·5 g. of average R.A. 6·2 from the Hawthorndale Laboratories of Imperial Chemical Industries Ltd.; stages (1) and (2) in the concentration were carried out at these places and the charcoal adsorbate was then worked up during 1940 in London and subsequently at Manchester.

Preliminary studies on the crude hatching factor

The crude solid obtained as above appeared to consist largely of a mixture of salts (mainly calcium) of organic acids. As a preliminary to concentration studies, the stability of the factor at various pH values was examined. The results obtained are shown in Table 1. It is evident that the factor, although moderately stable at pH 1–7 (allowing for some latitude in the biological assay values), is extremely rapidly deactivated at pH values above 7, both at 100° and at room temperature. In practice, the active principle in the crude solid frequently appeared to be much less stable than the values in Table 1 would suggest, activity being all too often lost for no apparent reason. This instability, coupled with the difficulty of accurate bioassay and the fact that the starting material was difficult to obtain in other than very small quantity, has throughout seriously hampered chemical investigation.

Table 1. *Stability of hatching factor in crude solid under varying* pH *conditions*

pH	Half life at 100° (min.)	Half life at 25° (hr.)
1·1	6	Very great
1·8	9	,,
2·7	15	,,
3·6	15	,,
4·8	8	,,
5·8	17	,,
6·8	8	,,
8·1	2·5	1·5
9·1	1·5	—
10·4	0·25	0·15
11·2	0·25	—
12·1	—	0·0

As the active principle appeared to be acidic, various attempts were made to effect purification by precipitation as a metallic salt. These failed, the salts being uniformly too soluble in ethanol and their preparation being accompanied as a rule by marked loss of activity which was not restored on acidification. The factor was evidently of low molecular weight, since it diffused through a collodion membrane, and no marked concentration was obtained by electrodialysis. The crude solid gave no characteristic colour reactions, and, although it gave a reaction with carbonyl reagents, the active principle itself did not seem to be ketonic, since treatment of a solution with Brady's reagent and removal of the small precipitate of 2:4-dinitrophenylhydrazone had no marked effect on its activity.

BIOASSAY OF THE HATCHING FACTOR

Before serious chemical investigation of the potato eelworm hatching factor could be undertaken it was necessary to devise a reliable bioassay method. The literature revealed no method meeting our requirements, and comparatively little was known of the factors influencing the hatching of cysts. The usual method employed was to place a number of soaked cysts in dishes (e.g. deep solid watch glasses), add root excretion from potatoes and count the larvae produced. Hatching was usually allowed to continue for several weeks. Triffitt (1930) followed this procedure using, as a rule, single dishes at 25°. Franklin (1940) stressed the use of several dishes for each test in order to make possible a statistical examination of results, and drew attention to the unreliability of methods then available. Gemmell (1940) criticized this technique and preferred to study the hatching of single cysts. Using a method of his own, in which about 100 single cysts are treated, he examined cysts obtained from different potato-growing areas and showed them to differ in their hatchability, although they were otherwise alike. In particular, he drew attention to the important fact that, although cysts may appear perfect and be full of embryonated eggs, on test a considerable number always fail to hatch. In Gemmell's experiments hatching was allowed to continue for some months.

For chemical tests, such methods as the above were too slow and unreliable. It was, therefore, decided to carry out a series of investigations aiming at a quantitative method for testing concentrates for hatching activity, which could be carried out in a few days at any time of the year. It seemed possible that this might be achieved, despite the seasonal variation in hatchability, reported by Triffitt (1930), if attention were paid to source and pretreatment of

eelworm cysts as well as to conditions of hatching. The cysts used were obtained from two sources; sample *A* from eelworm-infested soil in Hertfordshire and sample *B* from similar soil in Chat Moss, Lancashire. The preparation of hatching factor used throughout the experiments, and described as 'Standard Solid', was a sample of crude solid material obtained in early experiments on tomato-root excretion in which 5 g. charcoal were used for each litre of leachings, and the aqueous acetone eluate was evaporated to dryness. Several grams of this product were set aside for use as a standard preparation; under suitable conditions it gave good hatches with viable cysts at dilutions from 1/20,000 to 1/40,000. Sample *A* cysts were on the average smaller and hatched more rapidly than those in sample *B*, although the latter were quite satisfactory to work with, and were otherwise very similar to those of sample *A* in behaviour. The specific objective of the experiments carried out was a simple test method, and not an exhaustive study of all the important factors in cyst hatching.

Sample A cysts. The soil was supplied by the late Mr A. C. W. MacDonald of Winches Farm, St Albans, and was collected in the autumn so that most of the cysts were probably young. The cysts were isolated from the soil by a flotation method followed by rolling off on a Bristol board, as described by Fenwick (1940), and were stored at room temperature in a closed jar. When required for use, batches of cysts were soaked for 2–4 weeks in water at 24° and then sorted under a dissecting microscope by Miss M. Oliver. Viable cysts were selected by their appearance in conjunction with the results of dissections of small numbers of samples for this purpose, and were kept in water at 24° until used, except in experiments dealing with the effect of temperature of storage.

The hatching of single cysts was examined as described by Gemmell (1940), and the results of two experiments are given in Table 2.

From these results it is evident that not only did many apparently normal cysts fail to hatch, but there were also great variations in the rate of hatching. Excluding cysts which failed to hatch at all, larval counts varied from 1 to 195, while the standard deviation of individual results was considerably greater than the mean.

Sample B cysts. These cysts were isolated by the usual flotation method from soil obtained from Chat Moss, Lancashire, through the kindness of Dr R. Stewart and Dr V. E. Henderson of Manchester University. Collection was made in August 1941, from a field in which potatoes were growing (the fourth successive crop), so that the cysts were probably at least a year old. As they varied considerably in size, the cysts were graded into three groups by sieving, approximate numbers/sack of soil being: (1) held by 40 mesh sieve, 22,000; (2) held by 60, passed by 40, mesh sieve, 60,000; (3) held by 100, passed by 60, mesh sieve, 60,000. The cysts obtained were stored at about 23° in a desiccator over saturated K_2CO_3 solution giving a relative humidity of some 50%. The intermediate group (2) was selected for general use and is described as sample *B*. As the technique of sorting previously used was rather tedious, the following method was adopted. The cysts were placed in a dish of water, stirred thoroughly and left for 2 days, after which time those that floated were rejected. Those which sank were sorted under a large lens in a strong light, when viable cysts could be readily distinguished by their uniform light brown colour and resistance to pressure with a blunt glass needle. The selected cysts were normally kept in water at 22° and used after about 3 weeks.

Table 2. *Hatching of single cysts from Sample A*

	Exp. 1	Exp. 2
	45 cysts treated with 1 ml. Standard Solid, 1/40,000 in tap water, for 3 days at 24°	45 cysts treated with 1 ml. Standard Solid, 1/20,000 in tap water, for 3 days at 24°
Hatches ...	11, 8, 0, 11, 10, 9, 27, 33, 1, 10, 0, 1, 0, 16, 3, 13, 0, 34, 7, 1, 0, 0, 0, 1, 0, 21, 0, 2, 0, 1, 7, 36, 1, 34, 46, 2, 4, 0, 1, 2, 1, 51, 0, 1, 0	13, 73, 63, 15, 0, 1, 0, 0, 0, 12, 38, 1, 1, 19, 96, 4, 23, 0, 24, 41, 21, 46, 50, 24, 26, 16, 13, 1, 64, 1, 74, 25, 70, 0, 20, 195, 0, 1, 54, 0, 7, 34, 32, 0, 5
Total hatch	406	1203
S.D. among individual results	14	36
Mean hatch	9	27
S.E. of mean	±2·1	±5
Hatching no larvae (%)	29	20
Hatching only 1 larva (%)	20	13
S.E. of mean hatch on the basis of a count of 80 cysts	1·5 (=17% of mean hatch)	4 (=15% of mean hatch)

Table 3. *Hatching of single cysts from sample B*

(25 cysts treated with 1 ml. Standard Solid, 1/30,000 in tap water, for 3 days at 23°.)

Hatches	3, 0, 0, 15, 0, 7, 7, 7, 0, 4, 4, 4, 49, 5, 17, 0, 0, 0, 0, 0, 0, 43, 5, 4, 0
Total hatch	174
S.D.	13
Mean hatch	7±2·6
Hatching no larva (%)	44

On the basis of a count on 80 cysts, the S.E. of the mean would be 1·5, or ±21% of the mean hatch.

From Table 3 it is clear that the behaviour of sample *B* cysts is rather similar to that of sample *A*, and that the variable distribution of good and inert cysts among the dishes makes a high standard deviation inevitable.

It was noticed in the course of sorting the Chat Moss cysts that in the case of the smaller cysts (groups 2 and 3) approximately one-third appeared viable, whereas the proportion of good cysts in group 1 was much less and nearly 90 % were empty. It is of interest to note that a quantity of soil obtained from the same field 8 months later (after the potato crop had been harvested) was found to contain some 70 % more cysts/sack and there were virtually no large cysts present; the proportion of viable cysts was about the same as in sample *B*.

Earlier workers (e.g. Triffitt, 1930) have reported marked seasonal variation in ease of hatching. To check this, a portion of sample *B* cysts was set aside over saturated potassium carbonate under the roof tiles during the autumn and winter of 1941–2, when they were exposed to considerable variations in temperature, frost and snow being frequent. At intervals of about 1 month, samples were removed, sorted as above described and the viable ones soaked for 3 weeks at 22° and tested for hatchability by incubating in groups of 15 for 3 days at 23° with 1 ml. of Standard Solid solution (1/40,000). Simultaneous tests were made on samples from a second batch stored continuously at 23°. In both batches it was observed that cysts hatched fairly well (about 150 larvae from 15 cysts) in August, but this gradually fell until, by the end of November, very low hatches (10–20 from 15 cysts) were obtained. Hatching remained very difficult throughout December and January, and began to improve again thereafter. Since it was shown by both batches of cysts, the variation in hatchability is evidently seasonal and not due simply to temperature of storage. In later work on the eelworm hatching factor we have always observed this effect and, indeed, great difficulty has been encountered in doing any reliable testing of concentrates during the winter months.

In the above experiments, cysts were usually soaked in water for at least 3 weeks before a hatching test was made. This practice was adopted as a result of experiments on the effect of soaking on hatching. A batch of viable sample *B* cysts was placed in water, and samples were removed and tested with Standard Solid solution (1/30,000) from time to time. The results are given in Table 4. The actual hatches are low as the experiment was made in January when cysts are rather difficult to hatch, but the effect of soaking is evident.

Table 4. *Effect of soaking cysts*

Period of soaking (days)	Mean hatch/dish of 20 cysts (3 days at 23°)
2	0
8	1
15	12
18	12
21	71
23	53

The effect of temperature on hatching and on soaking was examined in other experiments. In one of these, sample *A* cysts were kept at 24° for some weeks and then incubated at various temperatures with Standard Solid (1/30,000). The results are shown in Table 5. In the experiment leading to the results in Table 6, sample *A* cysts were sorted after 3 days soaking, and batches were then kept soaking for 11 days at various temperatures before hatching in the usual manner. Although these experiments are only very rough (their extension, although desirable, was rather beyond the scope of our present researches), the importance of temperature emerges quite clearly.

Table 5. *Effect of temperature on hatching*

Temperature during hatching	Mean hatch/dish (15 cysts; 3 days)
6°	2
Room temp. (about 15°)	16
24°	608
37°	7

Table 6. *Effect of temperature of soaking*

Temperature of soaking	Mean hatch/dish (15 cysts; 3 days at 24°)
6°	4
Room temp. (about 15°)	270
24°	680

For setting up a rapid testing method experiments were carried out to determine the time required to give a suitable hatch and maximum reproducibility. The results of a typical experiment are summarized in Table 7.

Table 7. *Mean hatch per dish*

	Mean hatch per dish																Standard deviation	Mean hatch
Dish no. ...	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		
Day 1	111	48	29	18	125	61	51	87	33	6	85	80	142	75	31	135	42	70±10·5
2	239	230	160	70	316	236	251	341	250	75	255	275	325	315	145	350	85	233±21
3	274	325	210	104	420	285	346	540	305	125	356	316	431	403	207	463	120	319±30
4	340	263	206	119	415	287	300	552	333	152	385	377	425	365	227	570	127	332±32

Table 8. *Calculation of standard error of means using different numbers of dishes*

Day	Using 6 dishes of 15 cysts	Using 4 dishes of 15 cysts
1	17·1 or ±24% of hatch	21 or ±30% of hatch
2	34·7 or ±15% ,, ,,	42·5 or ±18% ,, ,,
3	49 or ±15% ,, ,,	60 or ±19% ,, ,,
4	52 or ±16% ,, ,,	63·5 or ±19% ,, ,,

In this experiment 16 dishes were prepared each containing 15 cysts (sample *A*) to which 1 ml. Standard Solid solution was added. The dishes were incubated at 23° and counts made after 1, 2, 3 and 4 days. Table 7 shows the hatches obtained in each dish together with the standard deviations of the means.

In Table 8 the results have been further subdivided to give standard deviations calculated for groups of six or four dishes. It will be seen that hatching had practically ceased by the third day and that the standard deviation had fallen to a steady proportion, about 10% for 16 dishes, by the second day. With six or four dishes the standard deviations were 15% or 18–19%. Although it was convenient to use conditions where the number of larvae were from 180 to 200/dish, Table 3 shows that the standard errors of means were similar when the counts were much lower. From the results given it will be seen that with three to four dishes containing a total of 45–100 cysts standard deviations of about 20% are to be expected. General experience confirms this. For the purpose of a reproducible test it is the number of cysts used that is important, and these should be distributed among three to four dishes. Experiments showed that increasing the volume of solution above 1 ml. had little effect, and 1 ml. was usually used with 15–30 cysts/dish.

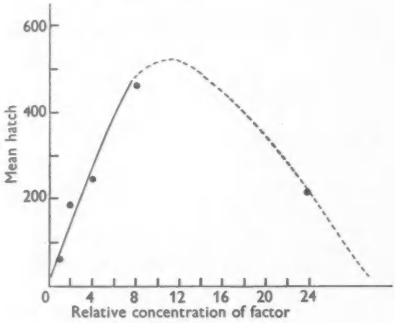


Fig. 8. Effect of hatching factor concentration on larval hatch.

A number of experiments was made on the effect of concentration of hatching factor on the hatching of cysts. In each case it was found that the number of larvae hatched was proportional to the concentration of factor up to a certain concentration, above which the hatching decreased. The result of one such experiment is shown graphically in Fig. 8. In the testing method which was finally adopted (see below) tests were always carried out on the first part of the curve, where concentrations are below those giving the maximum hatch, and it was assumed that the number of larvae which emerged was proportional to the concentration of the factor. It should, however, be noted that, on account of the high standard deviations usually obtained in proportion to the means, the hatch is a rather crude measure, even where a large number of dishes is used in each test (cf. Table 7). It has, nevertheless, served as the basis of the test method which we have used throughout the work on the eelworm hatching factor described in this and succeeding papers. The testing procedure is given in detail below.

Testing of materials for hatching activity

The practice adopted has been to compare the activity of unknown materials with a standard preparation. For this purpose a large sample of crude solid from tomato root leachings was set aside at an early stage in the investigations. This material was obtained by evaporating the eluate from a charcoal adsorbate prepared, using 5 g. charcoal/l. of leachings and is the material described as Standard Solid throughout the present paper. It gave good hatches from viable cysts at dilutions of 1/20,000 to 1/40,000 and was allotted a Relative Activity (R.A.) of 1.

Tests were carried out as follows: a suitable number of cysts (usually 20), previously sorted and kept in water for 3–4 weeks at 23°, was counted into each dish (deep solid watch glasses are convenient) using a Pasteur pipette; the water was drained off with a fine pipette and replaced by 1 ml. of the active solution. The dishes were now covered with squares of glass and incubated at 23–34° for 2–3 days, by which time 50–200 larvae had emerged in each dish. Counting was carried out directly under a low-power microscope. Never less than three dishes were used and where possible four or five were preferred both for the unknown and the standard.

It occasionally happened that single dishes showed counts markedly different from the others in the same group; it was customary to reject any count which was greater than five times, or less than one-fifth of, the other counts. Experience showed that the mean values were reasonably reliable, and when repeat estimations were carried out very similar results were usually obtained. After a test was completed, the cysts could be kept for a few days in water and used again; after several tests they tend to become mouldy and should then be discarded.

To estimate the activity of an unknown substance, it was dissolved in water at a suitable dilution and applied to one group of cysts, while to a control group a solution of Standard Solid at, say, 1/40,000 dilution

was added. After 2–3 days incubation, the emerged larvae were counted and from the result the R.A. of the unknown substance calculated.

In a typical experiment, an active concentrate was diluted to 1/2,000,000 and compared with Standard Solid at 1/40,000 with the following result:

Standard 1/40,000: hatches (2 days, 15 cysts/dish)—144, 57, 112, 150; mean 116.

Unknown 1/2,000,000: hatches (2 days, 15 cysts/dish)—165, 89, 163, 120; mean 134.

From the result the unknown substance had R.A.

$$\frac{2{,}000{,}000 \times 134}{40{,}000 \times 115} = 58.$$

It will be observed that a dilution was chosen (by trial and error) for the unknown substance which gave about the same hatch as the control. In these circumstances the importance of the ratio concentration/hatch was diminished, since two solutions giving the same hatch would be expected to contain the same concentration of factor.

In the past, workers have usually stated the maximum dilution at which materials show any activity. This is a very unsatisfactory method of expressing activity, partly because cysts vary in sensitivity and partly because it is difficult to decide the point at which hatching due to active material becomes significant, since even in water a few worms may hatch out. The test method we have described is easy to carry out and gives results quickly. Experience in many trials has shown that it is fairly reliable, and occasions on which it has given misleading results have been very few. Its accuracy is not very high, but the great individual variation among eelworm cysts makes it difficult to achieve precision in assay. In the work described in this and succeeding papers we have employed mainly cysts from Chat Moss, Lancashire, and from Cambridgeshire (for which we are indebted to Messrs F. G. W. Jones and F. R. Petherbridge); we have also used successfully cysts isolated from soil in the Newcastle area obtained through the kindness of Dr C. Ellenby.

SUMMARY

1. A method is described for the preparation of crude solid concentrates of the potato eelworm hatching factor by leaching the roots of growing tomato plants with water, adsorbing the factor on charcoal and eluting with aqueous acetone.

2. The effect of various conditions on the hatching of eelworm cysts has been studied, and a reasonably reliable bioassay method for the hatching factor has been worked out.

We are deeply indebted to Prof. R. T. Leiper, F.R.S., for his advice and assistance, to Imperial Chemical Industries Ltd. (Dyestuffs Division) and the Agricultural Research Council for their support of the investigations, and to Dr G. H. Orcutt of the Department of Applied Economics, Cambridge University, for statistical calculations.

REFERENCES

Baunacke, W. (1922). *Arb. biol. Abt.* (*Anst.—Reichsanst.*), *Berl.*, **11**, 185.

Buckhurst, A. S. & Fryer, J. C. F. (1930). *Ann. appl. Biol.* **18**, 584.

Ellenby, C. (1942). *Nature, Lond.*, **149**, 50.

Ellenby, C. (1945). *Ann. appl. Biol.* **32**, 237.

Fenwick, D. N. (1940). *J. Helminth.* **18**, 155.

Franklin, M. T. (1940). *J. Helminth.* **18**, 63.

Gemmell, A. R. (1940). *Bull. W. Scot. agric. Coll.* p. 139.

Hurst, R. H. (1935). *Agric. Res. Coun. Rep.* 1933–5, p. 50. London: H.M. Stationery Office.

Hurst, R. H. (1937). *Agric. Res. Coun. Rep.* 1935–37, p. 176. London: H.M. Stationery Office.

Massee, G. (1913). *Kew Bull.* p. 343.

Ministry of Agriculture and Fisheries (1947). Advisory Leaflet no. 307. London: H.M. Stationery Office.

Morgan, D. O. (1925). *J. Helminth.* **3**, 185.

O'Brien, D. G. & Prentice, E. G. (1930). *Scot. J. Agric.* **13**, 415.

Schacht, H. (1859). *Z. Ver. Rübenzucker Ind. Zollver.* **9**, 390.

Schmidt, A. (1871). *Z. Ver. Rübenzucker Ind. Zollver.* **21**, 1.

Smedley, E. M. (1939). *J. Helminth.* **17**, 31.

Triffitt, M. J. (1930). *J. Helminth.* **8**, 19.

Wollenweber, H. (1923). *Arb. Forsch. Inst. Kartoff., Berl.*, **7**, 52.

The Potato Eelworm Hatching Factor

2. PURIFICATION OF THE FACTOR BY ALKALOID SALT FRACTIONATION. ANHYDROTETRONIC ACID AS AN ARTIFICIAL HATCHING AGENT

By C. T. CALAM, A. R. TODD and W. S. WARING
Chemistry Department, University of Manchester, and Chemical Laboratory, University of Cambridge

(*Received* 8 *April* 1949)

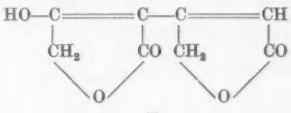
Methods for the preparation of crude solid concentrates of the potato eelworm hatching factor from leachings of tomato roots and for the bioassay of the factor being available as a result of work described in the preceding paper (Calam, Raistrick & Todd, 1949), experiments directed to the further concentration and eventual isolation of the active principle were undertaken. It was clear from preliminary experiments that, if only on account of a tendency to inactivation, attempted purification through metallic salts was unlikely to be successful, at least on the crude solid. Substantial purification could, however, be effected by extraction with organic solvents; thus from ether extracts of acidified aqueous solutions of crude solid a brownish glass was obtained having a Relative Activity (R.A.) about 25 and about one-quarter the weight of the starting material. (Relative Activity is defined by Calam *et al.* 1949, p. 518.) This step, which results in little loss in activity, was accordingly included in all later concentration procedures. The product was strongly acidic and gave on titration an equivalent value of about 200; addition of excess alkali and back titration indicated the presence of lactonic material. It contained small amounts of nitrogen, but was free from phosphorus, halogens or sulphur.

As the product obtained by ether extraction could not be crystallized, and was almost certainly a complex mixture, it was treated with various alkaloids and the alkaloidal salts subjected to fractional precipitation. Brucine and quinine proved the most suitable bases, although neither was very satisfactory, as there was frequently complete loss of activity. It was noticed, too, that when the R.A. of a brucine salt fraction had reached about 50 it was difficult to raise it further without heavy loss. Although most of the earlier work was done on brucine salts, it was later found that quinine salts were equally useful. Examples of fractionations with both alkaloids are given below. The products were amorphous, but had fairly sharp melting points and gave reasonably consistent analytical values. The purest brucine salts showed R.A. 60–65. It was observed on a number of occasions that inactivation of the most highly active brucine salt fractions, on standing, on precipitation with solvents or on treatment with a trace of alkali, yielded in nearly equivalent amount an inactive brucine salt which crystallized in rosettes of tiny needles and gave analytical values very close to those of the amorphous active salt. Such inactivation, with almost quantitative conversion to a crystalline salt, has not always been observed, perhaps because the amorphous active salts from different runs varied in their degree of heterogeneity. Treatment of the purified active brucine salts with acid, followed by continuous ether extraction, gave a highly acidic resin, active as hatching factor at dilutions of 1 in 10^7 to 1 in 10^8; a very similar, although biologically inactive, resin was obtained by the same treatment applied to the inactive crystalline salt. The properties of these materials, so far as they were examined, are recorded in the experimental section. The evidently heterogeneous products were so intractable, and the amounts available for study at any one time were so small, that it would be unwise to draw any conclusions beyond that the hatching factor—for which we propose the name *eclepic acid*—is an acid probably containing a lactone group. It was decided to defer further investigation of the natural material until larger amounts could be made available.

Since the hatching factor is obtained by leaching the roots of growing potato and tomato plants, a number of substances which might occur in plant roots were tested for possible hatching activity. They included aneurin, riboflavin, nicotinamide, pantothenic acid, pyridoxin, liver and yeast concentrates of the other B vitamins, ascorbic acid, heteroauxin, traumatic acid and a urine concentrate which was believed to contain auxins *a* and *b*; none of them showed any activity. Contrary to the statements of Hurst (1937), who was probably misled by the biological test method he used, tyrosine, glutamic acid, lysine picrate and picric acid were found to have negligible activity. The high acidity and probable lactonic nature of eclepic acid, however, led us to test also a number of tetronic acid derivatives and other compounds, viz. carolic acid (Clutterbuck, Raistrick & Reuter, 1935), tetronic acid (β-hydroxy-$\Delta^{\alpha:\beta}$-butenolide), α-methyltetronic acid, α-benzyltetronic acid, α-carboxymethyltetronic acid, α-acetyltetronic acid, α-bromotetronic acid,

methyl tetronate, *methyl α-methyltetronate*, *methyl α-benzyltetronate*, *acetyl α-benzyltetronate*, benzoyl α-methyltetronate, α-carbethoxytetronic acid, *α-carbethoxy-γ:γ-dimethyltetronic acid*, γ:γ-dimethyltetronic acid, α-bromo-α-methyltetronic acid, *O-tetra-acetyl-β-glucosidyl α-methyltetronate*. (Details of new compounds are given in the Experimental section.) None of these compounds showed any activity as hatching factors.

Anhydrotetronic acid, however, for which structure (I) has been established (Marrian, Russell, Todd & Waring, 1947), did show pronounced hatching factor activity at pH values below 3. The free acid at a dilution of 1/2000 was fully active and still showed distinct activity at a dilution of 1/8000. At a dilution of 1/2000, anhydrotetronic acid gives as large hatches as the best samples of natural hatching factor. This remarkable property is not shared by any of a number of close relatives of anhydrotetronic acid; *iso*propylidene-bis-tetronic acid (Wolff, 1901), ethylidene-bis-anhydrotetronic acid (Wolff, 1901) and bis-tetronic acid (Wolff, 1913) are all devoid of hatching activity.



(I)

The discovery that anhydrotetronic acid shows hatching properties similar to those of the natural factor, although the degree of activity on a weight basis was clearly very small compared with that of eclepic acid, led to systematic attempts to synthesize other active substances; these will be described in later communications. It also led to a preliminary study of the feasibility of artificially hatching eelworm cysts in sand. Although the experiment (described on p. 523) was on a small scale and the results only qualitative, there was a significant reduction in the larval content of cysts in sand which had been treated with anhydrotetronic acid.

EXPERIMENTAL

Concentration of eclepic acid by ether extraction. Crude solid eluate prepared from tomato leachings (Calam *et al.* 1949; 1 g., R.A. 6) was dissolved in dilute H_2SO_4 (7·5 ml. water + 1·5 ml. 2 N-H_2SO_4) and extracted continuously with peroxide-free ether for 12 hr. Evaporation of the ethereal extract followed by drying off *in vacuo* at room temperature gave a yellow acidic resin (212 mg., R.A. 28). Repetition of the procedure on many occasions using crude eluate of varying activity consistently gave products 3 to 4 times as active as the starting material. The product was free from S, P and halogens, but contained small amounts of N (1–2% estimated by the Dumas method); it was strongly acidic, behaving on titration as though it had an equivalent of about 200. Addition of excess alkali, warming and back titrating indicated the presence of an additional concealed acid group (probably a lactone group). Numerous attempts were made to prepare metallic salts from this material, but no crystalline products were obtained and extensive inactivation occurred. Brucine, cinchonine and quinine gave amorphous salts which appeared to retain a considerable proportion of the original hatching activity, and those from brucine and quinine were selected for further study.

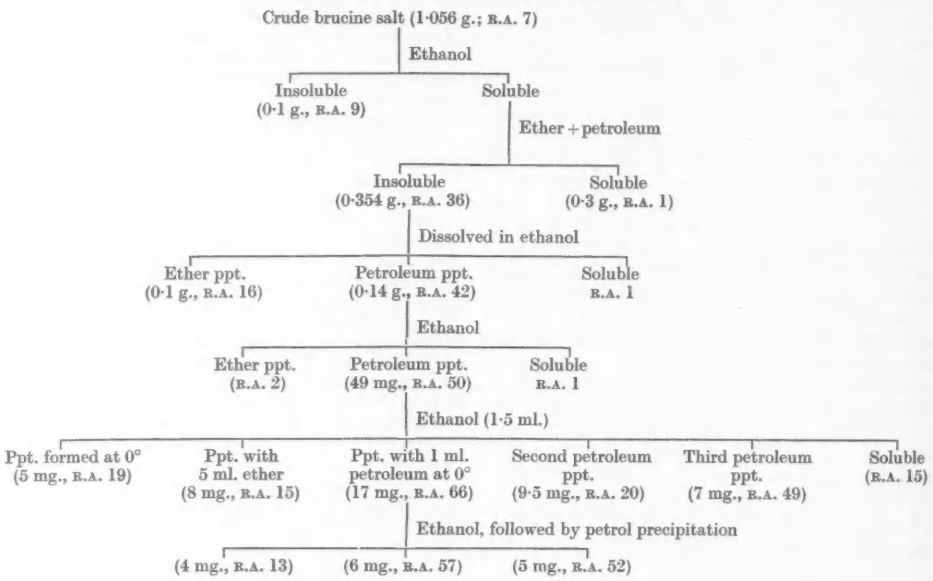
Fractionation of brucine salts. The above ether-free concentrate (0·15 g.) was dissolved in water (8 ml.), brucine (0·5 g.) added and the mixture shaken thoroughly. After 30 min. the solution was decanted and the residue washed with water. The combined solution and washings were filtered and evaporated to dryness *in vacuo*. The resulting crude brucine salt, which usually had R.A. 5–7, was ground under ethanol, when most of the active material dissolved, and the dissolved material was then subjected to repeated fractional precipitation by adding first ether and then light petroleum (b.p. 40–60°) and repeating the process. By this means a small amount of amorphous active salt (m.p. indefinite 165–190°) was finally obtained of R.A. 55–65, but the process involved heavy loss. Attempts to purify beyond this point usually resulted in inactivation, and the activity of the best fractions did not rise. A typical fractionation is set out in Table 1.

A sample of purified brucine salt, m.p. 180–183°, of R.A. 58 was dried over P_2O_5/0·1 mm. and analysed. (Found: C, 62·6; H, 6·5; N, 4·9%.) When the active material was kept in ethanolic or aqueous solution it lost its activity, and on several occasions a crystalline inactive salt could then be isolated in a yield of 60–90%. This inactive material separated from ethanol in rosettes of colourless needles of rather indefinite m.p. 180–200°. Analysis of a recrystallized sample, m.p. 198–204°, gave values very similar to those obtained for the active salt. (Found in material dried at 50°/0·1 mm. over P_2O_5: C, 63·5; H, 6·4; N, 5·2; loss on drying 4·0%.) If the active salt were reasonably pure this might suggest inactivation by isomerization.

Fractionation of quinine salts. The above concentrate prepared by acid ether extraction (0·15 g.) was treated directly with quinine (0·14 g.) in ethanol (3·5 ml.) giving a solution of pH 6. Ether (0·5 ml.) and light petroleum (11 ml.) were added and the precipitate (83 mg., R.A. 22), which contained practically all the activity, was spun off. This precipitate was stirred with acetone and the insoluble portion (R.A. 15) removed. Addition of ether gave a precipitate (R.A. 15) which was removed and the solution concentrated to small bulk and precipitated with light petroleum. The product (26 mg.) had R.A. 54 and repetition of the precipitation procedure gave a salt of R.A. 70. (Found in material dried at 35°/0·1 mm. over P_2O_5: C, 64·6; H, 7·1; N, 5·9%.) Material of this composition and of R.A. 70–90 could frequently be obtained by this procedure, but at times inactivation would occur during fractionation for no obvious reason.

Properties of eclepic acid prepared from active brucine salt. When the brucine salt was dissolved in water, acidified with H_2SO_4 and continuously extracted with ether, an extract was obtained which, on evaporation, gave eclepic acid as a yellowish gum. The preparation of the free acid was accompanied by some loss of activity, but the best specimens (R.A. about 200) showed full hatching activity (i.e. comparable to 'Standard Solid' (Calam *et al.* 1949) at 1/40,000) at a dilution of $1/10^7$. Direct titration gave an equivalent of 258, while addition of excess alkali and back titration showed an

Table 1. *Fractionation of crude active brucine salts*


Crude brucine salt (1·056 g.; R.A. 7)
Ethanol
Insoluble (0·1 g., R.A. 9)
Soluble
Ether + petroleum
Insoluble (0·354 g., R.A. 36)
Soluble (0·3 g., R.A. 1)
Dissolved in ethanol
Ether ppt. (0·1 g., R.A. 16)
Petroleum ppt. (0·14 g., R.A. 42)
Soluble R.A. 1
Ethanol
Ether ppt. (R.A. 2)
Petroleum ppt. (49 mg., R.A. 50)
Soluble R.A. 1
Ethanol (1·5 ml.)
Ppt. formed at 0° (5 mg., R.A. 19)
Ppt. with 5 ml. ether (8 mg., R.A. 15)
Ppt. with 1 ml. petroleum at 0° (17 mg., R.A. 66)
Second petroleum ppt. (9·5 mg., R.A. 20)
Third petroleum ppt. (7 mg., R.A. 49)
Soluble (R.A. 15)
Ethanol, followed by petrol precipitation
(4 mg., R.A. 13)
(6 mg., R.A. 57)
(5 mg., R.A. 52)

additional concealed acid group, possibly lactonic in nature. A molecular weight determination by the Barger method gave a value of about 300, but excessive dilution of the solutions which had to be used made the result unreliable. (Found in material dried at 50°/0·1 mm. over P_2O_5: C, 55·7; H, 5·7%.) The product gave no reaction for carbonyl groups, and catalytic hydrogenation gave a variable uptake of hydrogen corresponding to that required for 1 double bond in a molecular weight of 300–400. The free acid showed only very feeble optical activity in dilute aqueous solution ($[\alpha]_D$ about +1°).

Synthesis of tetronic acid derivatives

Methyl tetronate. Excess ethereal diazomethane was added to tetronic acid and when evolution of N_2 ceased the solution was evaporated. Recrystallization of the residue from benzene/light petroleum and finally from a small amount of benzene gave the *ester* as colourless needles, m.p. 67°. (Found: C, 52·3; H, 5·3. $C_5H_6O_3$ requires C, 52·6; H, 5·3%.)

Methyl α-methyltetronate. Methylation of α-methyltetronic acid with diazomethane in the usual manner gave the *methyl ester* as a colourless liquid which distilled at 160–170°/15 mm. (Found: C, 56·3; H, 6·4. $C_6H_8O_3$ requires C, 56·2; H, 6·3%.)

Methyl α-benzyltetronate. Methylation of α-benzyltetronic acid with diazomethane gave the *methyl ester* as a colourless liquid, b.p. 155–160°/5 × 10^{-3} mm. (Found: C, 70·1; H, 6·0. $C_{12}H_{12}O_3$ requires C, 70·6; H, 5·9%.)

Acetyl α-benzyltetronate. α-Benzyltetronic acid was acetylated by heating on the steam bath with excess acetic anhydride for 2 hr. Acetic anhydride and acetic acid were removed under reduced pressure and the residue recrystallized from cold methanol. The *acetyl derivative* formed colourless needles, m.p. 69–70°. (Found: C, 67·1; H, 5·4. $C_{13}H_{12}O_4$ requires C, 67·2; H, 5·2%.)

α-Carbethoxy-γ:γ-dimethyltetronic acid. Diethyl malonate (32 g.) dissolved in dry ether (100 ml.) was allowed to react with sodium wire (4·6 g.) overnight. To the cooled mixture α-bromo*iso*butyryl bromide (23 g.) dissolved in dry ether (100 ml.) was added; after 24 hr. dilute H_2SO_4 (100 ml. of 3N) was added and the aqueous layer separated and evaporated under reduced pressure at room temperature. The sticky solid was purified by precipitation from benzene solution with light petroleum and finally recrystallized from a minimum of water. The *acid* formed colourless needles, m.p. 83–84°. (Found: C, 53·6; H, 6·0. $C_8H_6O_5$ requires C, 54·0; H, 6·0%.)

O-Tetra-acetyl-β-glucosidyl α-methyltetronate. Dry powdered silver α-methyltetronate (2·4 g.) was added to a solution of α-acetobromoglucose (4·1 g.) in dry benzene (10 ml.), and the mixture shaken for 10 hr. AgBr was removed and the filtrate set aside. The *glucoside* slowly separated as colourless needles and, after recrystallization from benzene/light petroleum, had m.p. 160–161°. (Found: C, 51·6; H, 5·5. $C_{19}H_{24}O_{12}$ requires C, 51·3; H, 5·4%.)

The hatching of eelworm cysts by anhydrotetronic acid

Anhydrotetronic acid is strongly acidic and differs from eclepic acid in that it is only active at pH values below 3. The results of a typical experiment in which the behaviour of anhydrotetronic acid was compared with the natural substance are summarized in Table 2.

Table 2. *Hatching of eelworms by anhydrotetronic acid at different* pH *values*

(The hatches are means of three dishes, each containing thirteen cysts incubated 3 days at 22°.)

Anhydrotetronic acid (1/2000)		Standard solid (R.A. 1) from tomato root leachings (1/30,000)	
pH	Hatch (larvae)	pH	Hatch (larvae)
2·65	83	3·22	83
4·40	10	4·82	93
5·76	3	6·67	71
6·98	2	6·98	19
8·03	10	8·04	21

The structure (I) of anhydrotetronic acid gives no explanation for this behaviour. The absorption spectrum does not change on dissolution in alkali, and alkaline solutions become active once more on acidification. It would thus appear that the hatching mechanism can only be started by anhydrotetronic acid over a narrower pH range than by the natural substance. From the above experiment it can be seen that anhydrotetronic acid has an R.A. of about 0·07 compared with over 200 for eclepic acid.

When cysts are exposed to anhydrotetronic acid, the larvae emerge in the usual way (cf. Calam *et al.* 1949) being coiled and moving freely. This contrasts with the effect produced by certain other substances, such as bleaching powder solution or picric acid, which cause larvae to emerge in an angular, lifeless condition, and so can hardly be described as true hatching agents.

The results of an experiment in which eelworm cysts were mixed with sand and treated with anhydrotetronic acid for 35 days is summarized in Table 3.

Sand (100 ml.) washed with HCl and water was placed in glass jars and 100 cysts and 20 ml. water well mixed with it. The anhydrotetronic acid was dissolved in water and added after 1 week. The jars were kept at 15–20° in a room where they were not exposed to sunlight, and were occasionally watered to keep the contents moist. It was noticed that with higher concentrations of anhydrotetronic acid the upper layer of sand became tinged with purple. Soil could not be used because it was impossible with it to reduce the pH to 3 or less, and even with 600 mg. of anhydrotetronic acid per jar the sand mixture only just maintained this level. The jars were examined after 35 days.

It will be seen that where anhydrotetronic acid was used the cysts were no longer viable, so that the degree of hatching was perhaps less than it might otherwise have been. In another set of jars, which were kept for 92 days, even the untreated cysts were non-viable, suggesting that complicating factors may have been operative. Under normal conditions, cysts will continue to hatch for long periods without dying. Thus when 135 cysts containing 13,635 larvae were placed, for a few days, in hatching factor solution and water alternately, 6466 larvae emerged during the next 4 months and more were still slowly hatching. Dissection of a number of cysts at this stage showed that all had contributed some larvae, maximal and minimal numbers per cyst remaining being 223 and 36 at the start and 130 and 5 afterwards. It would appear that in the above experiment with sand some unknown factor was causing the death of the cysts.

Table 3. *Reduction in content of cysts by anhydrotetronic acid*

(Readings after 35 days; initial content, 101·6 larvae.)

Anhydrotetronic acid			Acid recovered					
(mg./jar)	(cwt./acre)	pH of extract	Titration (mg.)	Hatching test	Hatchability of recovered cysts	No. of cysts recovered	Larval content of recovered cysts	Mean reduction in larval content
0	0	4·2	0	–	+	84	97·9	
0	0	4·3	0			93	105·8	
0	0	4·3	0			52	103·0	
0	0	4·4	0			79	92·0	
Mean		**4·3**	0			**77**	**99·8**	**1·8**
50	6·7	3·75	27			84	97·6	
50	6·7	3·95	26	–	–	70	104·0	
50	6·7	3·85	30			84	90·3	
Mean		**3·85**	**28**			**79·3**	**97·3**	**4·3**
200	27	3·45	77			82	97·9	
200	27	3·5	98	±	–	93	93·1	
200	27	3·4	115			92	95·7	
Mean		**3·45**	**97**			**89**	**95·6**	**6·0**
600	80	2·85	300			105	77·3	
600	80	2·85	365	+ +	–	95	92·8	
600	80	2·75	362			107	88·5	
Mean		**2·8**	**342**			**102·3**	**86·2**	**15·4**

From Table 3 it will be seen that the reduction in number of larvae per cyst after 35 days was 1·8, 4·3, 6·0 and 15·4 with 0, 50, 200 and 600 mg. anhydrotetronic acid per jar. Statistical analysis shows that there is, in fact, a significant correlation between amount of anhydrotetronic acid added and the reduction in number of larvae. When a parallel series of jars were examined after 92 days a similar effect was observed.

SUMMARY

1. The potato eelworm hatching factor can be extracted with difficulty from acid aqueous solution by ether, and this process has been employed as a first stage in the purification of crude concentrates from tomato root leachings. The hatching factor is an acid, and for it the name eclepic acid is proposed.

2. The preparation of highly active brucine and quinine salts from eclepic acid concentrates is described, as also are some of the properties of the resinous free acid prepared from them. The most active preparations of eclepic acid, although still impure, show full hatching activity on eelworm cysts at a dilution of 1 in 10^7 to 1 in 10^8.

3. No appreciable hatching activity was shown by a variety of substances likely to occur in plant leachings or by several amino-acids and tetronic acids. Anhydrotetronic acid, however, was found to possess definite hatching properties, being fully active at a dilution of 1/2000. A preliminary pot experiment showed that it could bring about artificial hatching of eelworm cysts in sand.

Our thanks are due to the Imperial Chemical Industries Ltd. and to the Agricultural Research Council for the provision of grants and other assistance.

REFERENCES

Calam, C. T., Raistrick, H. & Todd, A. R. (1949). *Biochem. J.* **45**, 513.

Clutterbuck, P. W., Raistrick, H. & Reuter, F. (1935). *Biochem. J.* **29**, 300.

Hurst, R. H. (1937). *Agric. Res. Coun. Rep.*, 1935–37, p. 176. London: H.M. Stationery Office.

Marrian, D. H., Russell, P. B., Todd, A. R. & Waring, W. S. (1947). *J. Chem. Soc.* p. 1365.

Wolff, L. (1901). *Liebigs Ann.* **315**, 162.

Wolff, L. (1913). *Liebigs Ann.* **399**, 310.

The Potato Eelworm Hatching Factor

3. CONCENTRATION OF THE FACTOR BY CHROMATOGRAPHY. OBSERVATIONS ON THE NATURE OF ECLEPIC ACID

By D. H. MARRIAN, P. B. RUSSELL, A. R. TODD and W. S. WARING
Chemical Laboratory, University of Cambridge

(*Received* 8 *April* 1949)

From the preliminary investigations described in Part 2 of this series (Calam, Todd & Waring, 1949) it was clear that unless considerably larger amounts of starting material could be made available, little progress towards the isolation and structural elucidation of the potato eelworm hatching factor (eclepic acid) was likely. Although the factor evidently had a comparatively low molecular weight, the small amount of it present in tomato root leachings, coupled with its great instability, made chemical investigation extremely difficult. Fortunately, larger supplies became available through the generous help afforded by the Agricultural Research Council, which arranged for the growing and leaching of tomato plants on a large scale by essentially the method described in Part 1 (Calam, Raistrick & Todd, 1949) to be carried out at the Experimental Station, Cheshunt. There a stock of some 150,000 tomato plants was maintained under glass during the period May 1943 to September 1944. Production in 1944 was seriously affected by war damage at the station and ceased in the autumn of that year. The total amount of crude solid eluate obtained during the period was 218 g. with an average Relative Activity (R.A.) 9 (Calam, Raistrick & Todd, 1949), batches ranging from R.A. 4–13; under this arrangement then, we could extract about 12 g. crude solid per month, which, although small, was very much more than was available to us in our earlier work. The investigations described in this paper were all done on this material, referred to throughout as 'crude solid'. Assuming that R.A. about 200, the value shown by the most highly purified eclepic acid preparations obtained, represents something approaching the activity of pure eclepic acid, then on assay the total material we

received might contain 5–8 g. It has to be remembered, however, that the concentration procedure is always accompanied by heavy loss, and that the assumption regarding the activity of pure eclepic acid is wholly speculative, so that in fact the amount of factor obtainable in practice was much less than that which might be deduced from the bioassay figure for the crude solid.

From the early concentration studies of Calam, Todd and Waring (1949), it seemed clear that ether extraction from acid solution would be a valuable step in any isolation method, giving, as it does, a substantial reduction in weight without, as a rule, serious loss in activity. Accordingly, it was adopted as a standard first operation on crude solid in the present work. The alkaloid salt fractionation used in Part 2 of this series (Calam, Todd & Waring, 1949) was much less satisfactory, losses being heavy, and complete inactivation occurring at times for no obvious reason. It was, therefore, decided to examine adsorption methods more closely in the hope that one could be found which might effect a more far-reaching concentration of active material before fractional precipitation of salts was used. Preliminary experiments (by Dr C. T. Calam) had shown that simple chromatography on alumina was of little value, as was indeed to be expected from the general properties of the active material. Adsorption of active material from aqueous solution at neutral and acid pH on kieselguhr, kaolin, fuller's earth and charcoal was then examined; of these adsorbents only charcoal was really effective, even when the ratio of absorbent to material used was as high as 200/1. Attention was therefore turned to partition chromatography. At first the methods of Martin & Synge (1941) and Gordon, Martin & Synge (1943) were tried, using a silica gel column impregnated with methyl orange and applying to it the acid ether extract of the crude solid. Results were, however, unsatisfactory, as activity tended to spread over the column giving very indistinct banding. More success was achieved using a modification of this technique in which the chromatogram was a silica gel column loaded with strong potassium phosphate buffer at a suitable pH.

We are indebted to Dr A. A. Levi for allowing us to take advantage of this method, originally devised for work in the penicillin field, and as a result being unavailable through normal publication channels until later (Levi, 1948).

As a result of many trial experiments, a standard procedure of chromatography on a column buffered at pH 6 was adopted. The main active fractions from such chromatograms were resins showing a 3- to 4-fold increase in activity over the starting material. For further purification these products were treated with brucine, and the brucine salts shaken in aqueous solution with chloroform. The active salts passed into the chloroform layer and could be obtained as amorphous white powders by final precipitation from ethanolic solution with light petroleum. These salts gave reasonably consistent analytical results, as did the resinous eclepic acid preparations made from them.

This revised concentration procedure was found to be in general more reliable than the methods employed earlier, and the final products had R.A. 200–240 as free acid and R.A. 40–50 as brucine salts. Some of the brucine salts described in Part 2 (Calam, Todd & Waring, 1949) showed R.A. 60–65, but gave similar analytical results; since the tests were carried out at different periods and, bearing in mind the variability encountered in cyst hatching, we do not consider that this difference in R.A. is necessarily significant.

EXPERIMENTAL

Preparation of columns

In general, the method followed that described by Gordon *et al.* (1943). A weighed amount of the prepared silica (about 60 g./g. of substance to be chromatographed) was dried overnight at 110° in an electric oven, and then stirred in a large basin with half its weight of strong phosphate buffer (30%) of the appropriate pH, which was added dropwise from a dropping pipette. When all the buffer had been added, the silica was mechanically stirred with wet $CHCl_3$ until the small lumps had been broken up and then poured in the usual manner into a glass column plugged with a little cotton wool. The column was washed with wet $CHCl_3$ and then with wet ether. When the entire column had been washed with ether, the material to be chromatographed was added in wet ether.

Partition chromatography of eclepic acid concentrates

The material submitted to chromatography was in all cases the product obtained by continuous ether extraction of an acidified aqueous solution of 'crude solid' according to the method described by Calam, Todd & Waring (1949).

The most suitable pH for chromatography by the method of Levi (1948) depends on the stability of the substance under examination and its partition coefficient between buffer and solvent, and must be determined empirically. It was known from earlier work that eclepic acid was relatively stable below pH 7, and it was found by trial that 70–75% of the active material passed from ether into phosphate buffer of pH 5. This distribution appeared to be of the right order and, after trials on a series of columns at pH's in this region, pH 6 was finally selected as the most suitable. On a column of pH 6 most of the material was firmly held, only a pale yellow inactive oil passing through with ether; this inactive oil had a terpene-like odour and is referred to as the 'terpene fraction'. The chromatogram could be developed with acetone-ether mixtures. Experience showed that there was some spread of activity in the various fractions, but the majority of the active material was located in the 50% acetone-ether runnings. When all the active material had been eluted, the silica was yellow in colour and this colour—probably due to an anthoxanthin colouring matter—could

only be removed with water. Many such chromatograms were studied, and finally the following method was adopted in which only three fractions were taken. The material to be purified was put on the column in wet ether and the inactive terpene fraction (usually 30–35%) collected in the ether runnings and washings. The column was now pushed out of the tube and cut in half. The upper half, which contained all the activity, was eluted by stirring with acetone-ether (1:1) giving an active middle fraction (25–30%). The third, inactive, fraction containing yellow colouring matter could subsequently be eluted from the column with water. Typical results are given in Table 1. On some occasions there was found associated with the active fraction on the column a neutral biologically inactive crystalline compound, m.p. 136°. This substance appeared to have a formula $C_{12}H_{14}O_4$ on the basis of analysis and molecular weight determination, but its structure has not been determined.

Table 1. *Middle fractions from buffered chromatograms at* pH 6

Crude ether extract		Middle fraction		Wt. recovery (%)
Wt. (mg.)	R.A.	Wt. (mg.)	R.A.	
208	25	82	70	40
327	36	109	126	30
412	30	153	98	37
1193	—	422	68	37
2150	22	650	80	30

Purification of middle fractions from chromatograms

Attempts were now made to purify the eclepic acid present in the middle fractions from chromatograms of the type discussed above by brucine salt fractionation. After numerous trials it was found that virtually colourless active salts could be obtained by extracting the mixed brucine salts in aqueous solution with $CHCl_3$ in which the active salts are readily soluble and then re-extracting from the $CHCl_3$ layer with suitable quantities of water. Active salts obtained in this way were then further purified by precipitation from ethanol with ether-light petroleum mixtures. In this way amorphous brucine salts of R.A. 40–50 were obtained which gave consistent analyses. In the course of this work small amounts of three different inactive crystalline brucine salts were obtained; the first had m.p. 162–168° and appeared to be brucine formate, the second, m.p. 187–190°, probably brucine β-hydroxyglutamate and a third, m.p. 159–162°, which gave analytical values very similar to those given by the active salts.

From the purified active brucine salts, free eclepic acid was obtained as a yellowish resin by acidification and extraction with peroxide-free ether. This hygroscopic resinous product gave, like the parent brucine salts, fairly consistent analyses and usually had R.A. 200-240. During the preparation of the free acid, complete inactivation occasionally occurred; this unaccountable tendency to inactivation has been a major source of difficulty at all stages of work with the potato eelworm factor. The resinous acid defied all efforts at crystallization and could not be distilled without decomposition and loss of biological activity. A very large number of unsuccessful attempts was also made to obtain a crystalline salt or other derivative so as to get some evidence as to its homogeneity, e.g. Na, Ba, Ca, Ag, Pt, benzylamine, acridine, benzylthiuronium salts, *p*-phenylphenacyl and other esters, amides, anilides, toluidides, hydrazides. The acid gave a transient reddish brown colour with $FeCl_3$—a reaction which had apparently been missed in earlier work—but otherwise showed no characteristic colour reactions.

In Table 2 analytical figures are given for a series of active brucine salts, R.A. 40–50, and for the resinous eclepic acid preparations obtained from the salts. Agreement between individual analyses is surprisingly good considering the nature of the materials.

Table 2. *Analyses of amorphous active brucine salts and of the resinous eclepic acid prepared from them*

Sample no.	Brucine salt (dried over P_2O_5 at 100°/0·1 mm.)			Free acid (dried over P_2O_5 at 60°/0·1 mm.)		
	C (%)	H (%)	N (%)	C (%)	H (%)	N (%)
1	63·3	6·7	5·7	57·0	6·2	—
2	63·9	6·6	6·2	55·6	6·3	1·6
3	63·2	6·3	6·2	55·9	5·6	2·2
4	62·7	6·9	6·6	57·0	6·4	1·5
5	63·6	6·5	5·8	55·9	5·9	2·5
6	63·4	6·5	6·2	—	—	—
Mean	**63·4**	**6·6**	**6·1**	**56·4**	**6·1**	**2·0**

The low but ever-present amount of N in eclepic acid preparations was a continued source of confusion. Two possibilities had to be considered. The eclepic acid preparations, in view of their more or less consistent analyses and activity, might be nearly pure, in which case the mol. wt. would require to be of the order 700, or, alternatively, the N might be present in some contaminating material. On the whole, the second possibility seemed the more likely. Eclepic acid preparations showed no basic properties, were not inactivated by treatment with HNO_2 and yielded their N quantitatively in the form of NH_3 (identified as ammonium chloroplatinate) on hydrolysis at 100° with HCl. To throw further light on this point we decided to investigate the use of a second silica-phosphate buffer chromatogram at more acid pH in place of the brucine salt fractionation. It was found that the active material present in the middle fraction from a pH 6 chromatogram could be made to travel down a chromatogram buffered at pH 4–4·5 if applied in ether containing 1% *n*-butanol and developed with ether containing increasing amounts of *n*-butanol. The most active fractions (R.A. about 200) from such chromatograms were those eluted with ether containing 4% *n*-butanol and they had N contents of 1% or even less. It is thus very tentatively concluded that eclepic acid is N-free, it being assumed on available evidence that the purest preparations obtained, although certainly not homogeneous, consist largely of the factor. The only other possibility is that the active material is a minor constituent of the preparations obtained; this seems rather unlikely, particularly since these preparations are fully active at dilutions as high as 1 in 10^7.

DISCUSSION

In the absence of any real evidence for the homogeneity of the most highly purified samples of eclepic acid so far prepared, the interpretation of analytical or degradative data obtained using them is so

difficult that any conclusions can only be accepted with the greatest reserve. It is evident, from the analytical figures given for the free acid and its brucine salt in Table 2, that the material prepared by chromatography and salt fractionation was not homogeneous, but working on the assumption that it consisted largely of eclepic acid with minor amounts of impurity, we have carried out analytical and degradative experiments in the hope of finding some clue to the structure of the hatching factor. This assumption rests, of course, on the very high biological activity of the products and the fact that the carbon and hydrogen contents of samples prepared at different times from different batches of starting material were of the same order.

In general, titration of such materials indicated an equivalent of 250–290 and addition of excess alkali and back titration showed the presence of a lactone group (about 80 % of theory calculated on an equivalent of 250). From the ease of opening, the group concerned seems likely to be a γ-lactonic group and the hydroxyl involved in its formation is not tertiary; the greater resistance of lactone rings involving tertiary hydroxyls was checked by parallel examination of a series of synthetic compounds of this type. Unfortunately, the solubility of eclepic acid prevented determination of molecular weight by the common methods. A rather unsatisfactory attempt on one sample using Barger's method gave a value of about 300. Catalytic hydrogenation using both platinum and palladium catalysts led to hydrogen uptake corresponding to one double bond in a molecular weight of 300–400 according to the sample of material used. The optical activity of the purified materials was similar to that determined for earlier samples in Part 2, but all purified concentrates gave a reddish brown colour with ethanolic ferric chloride suggesting the presence of an enolic (or phenolic) grouping. Quantitative acetylation gave rather variable results indicating one to two hydroxyl groups in a molecular weight of 300–400. Zerewitinoff determinations indicated two to three active hydrogen atoms in the same molecular weight range. The absorption spectrum of eclepic acid concentrates was not very characteristic; in ethanolic solution the absorption maximum which could not be accurately determined occurred below 2200 A. ($E^{1\%}_{1\,cm.} > 30$), and there was a marked inflexion at 2850 A. (determination by Dr A. E. Gillam, Manchester University). These findings would not be inconsistent with the presence of a double bond in conjugation with a carboxyl and possibly an aromatic ring elsewhere in the molecule.

A variety of degradative experiments was carried out. Acid hydrolysis at various temperatures yielded no identifiable products other than traces of acetone, and alkaline hydrolysis was equally unsuccessful. The only identifiable product on oxidation with alkaline potassium permanganate or chromic acid was acetic acid, whilst selenium dehydrogenation gave only intractable resins. Treatment with diazomethane yielded products containing nitrogen, presumably by addition of the reagent to some unsaturated centre, and the methyl ester prepared from the silver salt with methyl iodide could not be satisfactorily purified. Methylation with dimethyl sulphate in alkaline solution at 70° yielded more promising results; the product could not be crystallized, but it gave analytical figures corresponding to a formula $C_{19}H_{26}O_8$. (Found in two different samples: C, 59·7; 59·1; H, 6·8, 6·4; MeO, 8·3. $C_{19}H_{26}O_8$ requires C, 59·7; H, 6·8; 1MeO, 8·1 %.) The methylated acid shows no ferric chloride reaction and is free from nitrogen. It is of some interest to note that if the formula $C_{19}H_{26}O_8$ can be applied to this material it would be the methylation product of an acid $C_{18}H_{24}O_8$ whose carbon and hydrogen contents (C, 58·7; H, 6·5 %) are not widely different from those of our best eclepic acid preparations. Such a formula would be in reasonable accord with such evidence as is available regarding the molecular weight of the hatching factor and could accommodate the necessary carboxyl group, lactone group, double bond and two hydroxyls, of which one has acidic properties.

Some further degradative experiments have been carried out with the methylated acid described above, but discussion of them is deferred since, until an eclepic acid preparation is obtained which is known to be homogeneous, arguments based on degradative evidence may be very misleading and little weight can be attached to any formula for eclepic acid based on them. It is hoped that fresh supplies of raw material will enable us to prepare a pure specimen of eclepic acid so that progress can be made in its structural elucidation.

SUMMARY

1. The application of two-phase chromatography on silica gel loaded with strong phosphate buffer to the concentration of the potato eelworm hatching factor is described. Chromatography at pH 6 followed by brucine salt fractionation, or two-stage chromatography at pH 5·5–6 and 4–4·5, effect similar degrees of concentration, the resinous products being fully active in hatching eelworm cysts at dilutions of 1 in 10^7.

2. The composition and chemical properties of the most highly purified hatching factor concentrates are discussed.

Grants in aid of this work from the Agricultural Research Council and Imperial Chemical Industries Ltd. (Dyestuffs Division) are gratefully acknowledged. We are also greatly indebted to Dr Owen of the Cheshunt Experimental Station for his invaluable work in growing and leaching the tomato plants necessary for the investigation.

REFERENCES

Calam, C. T., Raistrick, H. & Todd, A. R. (1949). *Biochem. J.* **45**, 513.
Calam, C. T., Todd, A. R. & Waring, W. S. (1949). *Biochem. J.* **45**, 520.
Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1943). *Biochem. J.* **37**, 79.
Levi, A. A. (1948). *Biochem. J.* **43**, 257.
Martin, A. J. P. & Synge, R. L. M. (1941). *Biochem. J.* **35**, 1358.

The Potato Eelworm Hatching Factor

4. *SOLANUM NIGRUM* AS A SOURCE OF THE POTATO EELWORM HATCHING FACTOR

By P. B. RUSSELL, A. R. TODD and W. S. WARING
Chemistry Department, University of Manchester, and Chemical Laboratory, University of Cambridge

(*Received* 8 *April* 1949)

It has been known for some time that the root excretion of several species of *Solanum* stimulates the hatching of larvae from the cysts of the potato eelworm, *Heterodera rostochiensis* (Franklin, 1940). The isolation of the active principle in root excretion and the investigation of its chemical nature was first attempted, using the leachings from potato plants (Hurst, 1935, 1937), but the method of production was cumbersome and a more convenient source of material was found in tomato plants. The tomato plants were more easily grown indoors than potatoes, and gave a very active excretion. As work on the chemical nature of the active principle developed (Calam, Raistrick & Todd, 1949; Marrian, Russell, Todd & Waring, 1949), greater demands were made upon production of starting material for the investigation, and necessitated the growing of many thousands of tomato plants during a season. It became clear that if the scale of the work continued to expand a new source of root excretion, which could be easily and cheaply grown out of doors on a large scale, would be welcomed to replace the growing of tomato plants under glass. With this end in view, a small scale outdoor experiment with *Solanum nigrum* was carried out during 1943 at the suggestion of Sir E. J. Salisbury, F.R.S., who also very kindly supplied the seeds, and the yield, quality and nature of the root excretion were examined.

METHODS

The growing and leaching of Solanum nigrum *plants*

The plants were grown from seed in two trays *A* and *B* (each 4 × 3 ft.) having an inclined base of asbestos board which facilitated rapid drainage of leachings into a gutter of the same material leading to a large collecting jar. A layer of broken brick (½ in. deep) was laid at the bottom of each tray, and this was covered with a mixture of garden soil and sand (tray *A* contained 2 parts soil to 1 part sand to a depth of 4–5 in. and tray *B* 4 parts soil to 1 of sand). The seeds were sown in April 1943, in rows 5 in. apart, and the seedlings thinned out 6 weeks later, leaving about 120–130 plants in each tray. Towards the end of June, when the plants were 4 in. high and growing vigorously, they were well watered and a sample of leachings tested for activity by the method already described (Calam *et al.* 1949). The leachings proved to be highly active, hatching 683 larvae from 60 cysts during 3 days. It was therefore decided to begin regular leaching of the plants at once.

The plants were leached early in the morning on alternate days, and the leachings treated at once with activated charcoal (British Drug Houses Ltd., 'for decolorizing purposes', 1 g./l.), shaken and left to settle. On the following day the bulk of the liquid was decanted from the charcoal, which was then sucked fairly dry on a Buchner funnel. The moist charcoal was allowed to accumulate until the end of each week and was then eluted with aqueous acetone. Watering was controlled so as to give about 3–4 l. of leachings from each tray every alternate day. In actual practice, however, this volume was rather variable as the plants were unprotected from the weather, and during rainy periods the trays were liable to be almost completely depleted of their content of hatching factor. The leachings were a very pale yellow and had pH 7.

RESULTS

Elution of charcoal

The accumulated moist charcoal on which the active factor was adsorbed was eluted each week with aqueous acetone (70 parts acetone to 30 parts water). About 40 g. of charcoal were stirred four times with 100 ml. aqueous acetone and the yellow filtrate concentrated below 30° under reduced pressure, and finally evaporated to dryness in a desiccator. The

crude solid eluate was a brown resinous powder and had a high activity when tested against an arbitrarily chosen 'standard solid' eluate from tomato plants (cf. Calam *et al.* 1949). As will be seen from Table 1 the Relative Activity (R.A.) of the crude eluate from tray *A* was very high during the early period of growth (R.A. 20–30) and gradually decreased after the plants had reached maturity. The weight yield of crude eluate per litre of leachings also showed a gradual decrease throughout the experiment, except in the last recorded week which showed a large increase in weight yield. As this increase occurred in the case of both trays at the same date, it was probably due to the exceptionally heavy rainfall during the previous week. The wet weather during September brought the experiment to a premature close, although it is doubtful whether much more could have been expected from the plants even if the weather had been more favourable.

The yield of crude solid from tray *A* was better in weight and activity than that from tray *B* (Tables 1 and 2). This may have been due to a variety of reasons. Tray *A* was planted some 3 weeks earlier than tray *B* and contained rather more plants. The plants in tray *A* therefore had a more favourable growing period as the later part of the summer was rather wet and cold. Another difference between the two trays was that *B* contained less sand than *A*, and consequently drained more slowly. The total yield of crude eluate obtained from both trays (251 plants) during the experiment was 2·3 g.

Concentration of hatching factor

The purification of the crude solid eluate was carried out by applying the procedure which had been developed for the concentration of root excretion of tomato plants by Marrian *et al.* (1949).

The crude solid (2·3 g.) was dissolved in water, acidified with dilute H_2SO_4 and extracted continuously with peroxide-free ether for 12 hr. The moist ether extract (150 ml.) was put directly on to a column prepared from 20 g. silica gel moistened with 8 ml. 30% phosphate buffer, pH 6·0. After washing with wet ether (150 ml.) the column was drained and eluted by stirring with aqueous acetone (1:1). Evaporation of the ether runnings and washings gave 90 mg. of sweet smelling oil, probably terpene material, while the acetone eluate yielded 75 mg. of clear yellow resin, R.A. 150.

Brucine salt fractionation. The active concentrate from the chromatogram was dissolved in water (10 ml.) and shaken with brucine (250 mg.). The undissolved brucine (100 mg.) was filtered off, and the filtrate extracted by shaking several times with $CHCl_3$. Evaporation of the $CHCl_3$ extract gave 220 mg. brucine salts which were dissolved in ethanol (2·5 ml.) and a little insoluble matter spun off. Addition of ether caused no precipitate, so a large excess of light petroleum was added (20 ml.) and the precipitated brucine salt (63 mg.) was collected and analysed, R.A. 35. (Found in material dried *in vacuo* over P_2O_5 at 100°: C, 65·1; H, 6·5; N, 5·8%. Average value for corresponding product from tomatoes: C, 63·4; H, 6·6; N, 6·1%.)

Preparation of the inactivated free acid. The purified brucine salt (60 mg.) was dissolved in water, acidified with dilute H_2SO_4 and extracted continuously with ether for 20 hr. Evaporation of the ether extract left 16 mg. of free acid which

Table 1. *Details of hatching factor production from* Solanum nigrum (*tray A*)

Week ending	Volume of leachings (l.)	Wt. moist charcoal (g.)	Wt. crude eluate (mg.)	R.A.	Wt. crude eluate (mg./l.)
8 July 1943	11·5	42	205	24	18
17 July 1943	12	28	130	28	11
23 July 1943	13·5	38	160	32	12
31 July 1943	15·5	41	133	10	9
6 Aug. 1943	18	42	158	20	9
14 Aug. 1943	18	50	161	7	9
25 Aug. 1943	27·5	75	173	7	6
	(Heavy rain all week ending 1 Sept 1943)				
13 Sept. 1943	19	50	238	11	13
		Total	1358		

Table 2. *Details of hatching factor production from* Solanum nigrum (*tray B*)

Week ending	Volume of leachings (l.)	Wt. moist charcoal (g.)	Wt. crude eluate (mg.)	R.A.	Wt. crude eluate (mg./l.)
24 July 1943	9	26	123	—	14
30 July 1943	17	57	183	18	11
6 Aug. 1943	11	26	103	10	9
14 Aug. 1943	14·5	40	137	3	9
25 Aug. 1943	27·5	81	156	3	6
	(Heavy rain all week ending 1 Sept. 1943)				
13 Sept. 1943	17	55	241	5	14
		Total	943		

was inactive. (Found in material dried *in vacuo* over P_2O_5 at 50°: C, 60·0; H, 6·1; N, 2·0%. Average value for corresponding product from tomatoes: C, 56·4; H, 6·1; N, 1·8%.)

CONCLUSIONS

The growing of *Solanum nigrum* out of doors with the minimum amount of attention yielded a root excretion which was highly active in hatching larvae of the potato eelworm from their cysts. Unfortunately, the rapid growth and short growing period of the plants did not permit more than about 9 weeks' leachings to be collected. The active principle from *S. nigrum* root excretion could be concentrated by the same procedure as was used for tomato root excretion, but the limited number of analyses on the brucine salt and free acid did not justify the deduction that the active principle from *S. nigrum* was identical with that obtained from tomatoes. Nevertheless, making allowance for the non-crystalline character of the products, the great similarity in general properties would make it appear probable that the *S. nigrum* factor is either identical with that obtained from tomatoes or some very closely related substance.

SUMMARY

1. *Solanum nigrum* grown out of doors yielded a root excretion which was highly active in hatching potato eelworm cysts.

2. Concentration of the active material in this excretion gave a product which was closely similar to the corresponding product from tomato root excretion in its properties. The active principle in the two cases may well be identical.

We are grateful to Imperial Chemical Industries Ltd., and to the Agricultural Research Council, for grants in aid of this work. We wish to thank Sir E. J. Salisbury, F.R.S., for his interest and advice.

REFERENCES

Calam, C. T., Raistrick, H. & Todd, A. R. (1949). *Biochem. J.* **45**. 513.

Franklin, M. T. (1940). *J. Helminth.* **18**, 68.

Hurst, R. H. (1935). *Agric. Res. Coun. Rep.* 1933–5, p. 50. London: H.M. Stationery Office.

Hurst, R. H. (1937). *Agric. Res. Coun. Rep.* 1935–7, p. 176. London: H.M. Stationery Office.

Marrian, D. H., Russell, P. B., Todd, A. R. & Waring, W. S. (1949). *Biochem. J.* **45**, 524.

The Potato Eelworm Hatching Factor

5. ATTEMPTS TO PREPARE ARTIFICIAL HATCHING AGENTS. PART I. SOME FURAN DERIVATIVES

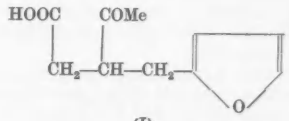
By P. B. RUSSELL, A. R. TODD and W. S. WARING
Chemical Laboratory, University of Cambridge

(*Received* 8 *April* 1949)

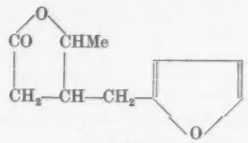
From the time when it became clear that highly active concentrates of the potato eelworm hatching factor prepared from tomato root excretion possesses both acidic and lactonic properties (Calam, Todd & Waring, 1949), synthetic experiments have been carried out alongside work on the natural factor in the hope that simpler substances might be prepared which would show hatching activity and which might be of practical interest in controlling eelworm infestation in potato crops. The examination of a number of tetronic acid derivatives has been described in a previous paper (Calam, Todd & Waring, 1949); the present communication deals with experiments on the synthesis of a number of lactones containing furan nuclei, such compounds being of interest, since it seemed probable that the natural hatching factor contained heterocyclic oxygen.

In commencing studies in the furan series an attempt was made to oxidize furitaconic acid (furfurylidenesuccinic acid) to β-hydroxy-β-carboxy-γ-2-furylbutanolide by means of potassium permanganate or monoperphthalic acid without result; this was unexpected in view of the ease with which itaconic acid yields hydroxyparaconic acid on similar treatment. Condensation of furfural with laevulinic acid in presence of sodium acetate gives a 10% yield of β-furfurylidene-laevulinic acid (Kehrer & Kleberg, 1893). Replacement of sodium acetate by piperidine failed to give any condensation and sodium hydroxide gave sticky products; with potassium acetate, however, yields of about 30% were obtained. No condensation occurred between furfural and ethyl laevulinate in presence of piperidine, but with sodium hydroxide β-furfurylidene-

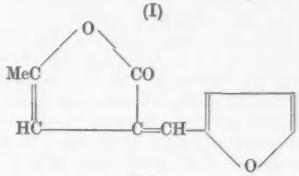
laevulinic acid was produced in moderate yield. Reduction of β-furfurylidene-laevulinic acid with sodium amalgam gave, according to conditions, *β-furfuryl-laevulinic acid* (I) or *β-furfuryl-γ-methylbutanolide* (II). Attempts to prepare analogous compounds by condensing ethyl 5-methyl-2-formylfuran-4-carboxylate with laevulinic acid failed.



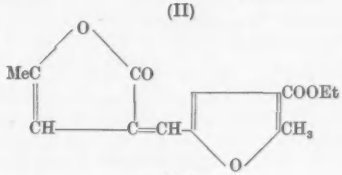
(I)



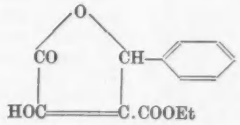
(II)



(III)



(IV)



(V)

Condensation of furfural with $\Delta^{\beta:\gamma}$-angelicalactone gave *α-furfurylidene-γ-methyl-$\Delta^{\beta:\gamma}$-butenolide* (III), hydrolysed to *α-furfurylidene-laevulinic acid* and reduced by sodium amalgam to *α-furfuryl-γ-methylbutanolide*. On refluxing α-furfurylidene-laevulinic acid with acetic anhydride it was reconverted to (III) in good yield. In similar fashion ethyl 5-methyl-2-formylfuran-4-carboxylate and $\Delta^{\beta:\gamma}$-angelicalactone gave *α-(5-methyl-4-carbethoxyfurfurylidene)-γ-methyl-$\Delta^{\beta:\gamma}$-butenolide* (IV). This lactone was readily hydrolysed to *α-(5-methyl-4-carboxyfurfurylidene)-laevulinic acid*, which gave *α-(5-methyl-4-carboxyfurfurylidene)-γ-methyl-$\Delta^{\beta:\gamma}$-butenolide* on refluxing with acetic anhydride. On reduction with sodium amalgam the original lactone (IV) gave as the only pure product *α-(5-methyl-4-carboxyfurfuryl)-laevulinic acid*. Attempts to condense furfural with ethyl sodio-oxaloacetate gave only resinous products, although benzaldehyde condensed readily enough, yielding the crystalline *α-hydroxy-β-carbethoxy-γ-phenyl-$\Delta^{\alpha:\beta}$-butenolide* (V; cf. Gault & Durand, 1943).

All the above lactones and the corresponding laevulinic acid derivatives were tested for hatching activity on potato eelworm cysts by the technique described elsewhere (Calam, Raistrick & Todd, 1949), but in no case was any activity detected. Further synthetic work in this series was therefore abandoned, especially as the discovery that anhydrotetronic acid shows hatching activity had suggested other possible lines of development.

EXPERIMENTAL

β-Furfuryl-laevulinic acid. β-Furfurylidene-laevulinic acid (2 g.) was dissolved in aqueous NaOH (10 ml. of 3 N), diluted with water (200 ml.) and Na amalgam (50 g. of 4%; 2 mol.) added with shaking. After a time the solution became colourless and it was then acidified, extracted with ether and the extract dried and evaporated. The crystalline product was purified by sublimation at 70°/10^{-4} mm. (bath temp.). Colourless needles, m.p. 98–99°. (Found: C, 60·8; H, 6·2. $C_{10}H_{12}O_4$ requires C, 61·2; H, 6·1%.)

β-Furfuryl-γ-methylbutanolide. β-Furfurylidene-laevulinic acid was dissolved in aqueous NaOH (50 ml. of 3 N) and excess Na amalgam (150 g. of 4%; 6 mol.) added. The mixture, which became colourless in 1 hr., was shaken overnight and then acidified and extracted with ether. The ethereal extract was thoroughly washed with $NaHCO_3$ and water, dried and evaporated. The residual *lactone* (1·8 g.), a colourless oil, was purified by distillation and had b.p. 70–75°/10^{-1} mm. (bath temp.); at atmospheric pressure it had b.p. 230°. (Found: C, 66·2; H, 6·7; equiv. (lactone), 183. $C_{10}H_{12}O_3$ requires C, 66·7; H, 6·7%; equiv. (lactone), 180.) On refluxing the lactone with hydrazine hydrate in ethanol it yielded the hydrazide of γ-hydroxy-β-furfurylvaleric acid, crystallizing from ethyl acetate as colourless plates, m.p. 153–134°. (Found: N, 13·5. $C_{10}H_{16}O_3N_2$ requires N, 13·2%.)

α-Furfurylidene-γ-methyl-$\Delta^{\beta:\gamma}$-butenolide. A mixture of furfural (1 g.), $\Delta^{\beta:\gamma}$-angelicalactone (1 g.) and piperidine (5 drops) was warmed on the steam bath for 1 hr. and then allowed to cool. The yellow solid was washed with a little ether and recrystallized from methanol. The yellow needles, m.p. 78–79°, had a slight brownish tinge which could be removed by sublimation *in vacuo*. (Found: C, 68·0; H, 4·7. $C_9H_8O_3$ requires C, 68·1; H, 4·5%.)

α-Furfurylidene-laevulinic acid. The above lactone (1 g.) was refluxed with excess methanolic KOH (3%) during 3 hr. Most of the methanol was then distilled off and the residue diluted with water and acidified. The yellow oil which separated was dissolved in a minimum of hot aqueous ethanol. On cooling *α-furfurylidene-laevulinic acid* separated as colourless needles, m.p. 125–126°. (Found: C, 62·3; H, 5·4. $C_{10}H_{10}O_4$ requires C, 61·8; H, 5·1%.) On refluxing with acetic anhydride (3 ml.) for 2 hr. the acid (0·1 g.) was reconverted to the above butenolide (0·09 g.), m.p. 78–79°.

α-Furfuryl-γ-methylbutanolide. The above unsaturated lactone (2 g.) was dissolved in aqueous ethanol (50 ml. of 90%) and Na amalgam (150 g. of 4%; 6 mol.) added. The mixture was shaken until colourless (about 1 hr.) then acidified and ethanol removed by evaporation under reduced pressure. The residual solution was extracted with ether and the extract washed with $NaHCO_3$, dried and evaporated. The pale yellow residual oil distilled at 85°/10^{-1} mm. (bath temp.). (Found: C, 66·4; H, 6·5; equiv. (lactone), 180. $C_{10}H_{12}O_3$ requires C, 66·7; H, 6·7%; equiv. (lactone), 180.)

Refluxed with hydrazine hydrate in ethanol the product gave the *hydrazide* of γ-hydroxy-α-furfurylvaleric acid as colourless needles (ethyl acetate), m.p. 138–139°. (Found: C, 56·6; H, 7·5; N, 13·6. $C_{10}H_{16}O_3N_2$ requires C, 56·6; H, 7·5; N, 13·2%.)

Ethyl 5-methyl-2-formylfuran-4-carboxylate. Prepared by oxidation of ethyl 5-methyl-2-(*d*-arabotetrahydroxybutyl)-furan-4-carboxylate (West, 1925; Gonzalez, 1934), the aldehyde had m.p. 56° and gave a 2:4-dinitrophenylhydrazone, m.p. 220–221°. Unknown to us at the time this work was carried out, it had also been prepared by Müller & Varga (1939), and was subsequently described by Jones (1945).

α-(5-Methyl-4-carbethoxyfurfurylidene)-γ-methyl-$\Delta^{\beta:\gamma}$-butenolide. Prepared in similar fashion to the analogous α-furfurylidene lactone by condensing $\Delta^{\beta:\gamma}$-angelicalactone with ethyl 5-methyl-2-formylfuran-4-carboxylate. Yellow needles, m.p. 143–144° from methanol. (Found: C, 63·8; H, 5·4. $C_{14}H_{14}O_5$ requires C, 64·1; H, 5·3%.) Reduction in aqueous ethanol with Na amalgam in the usual manner gave *α-(5-Methyl-4-carboxyfurfuryl)-laevulinic acid* crystallizing from water in colourless needles, m.p. 126–129°. (Found: C, 56·4; H, 5·6. $C_{12}H_{14}O_6$ requires C, 56·7; H, 5·5%.) Treatment of the gummy neutral fraction (which gave a positive Legal reaction) from the reduction mixture with hydrazine gave a colourless product, m.p. 218–219°, which from analysis appeared to be the *pyridazine* corresponding to α-(5-methyl-4-carboxyfurfuryl)-laevulinic acid. (Found: C, 57·7; H, 5·5; N, 11·0. $C_{12}H_{14}O_4N_2$ requires C, 57·6; H, 5·6; N, 11·2%.)

α-(5-Methyl-4-carboxyfurfurylidene)-laevulinic acid. The foregoing ester lactone (0·5 g.) was refluxed with methanolic KOH (3%) for 3 hr., the bulk of the methanol removed under reduced pressure and the residue diluted with water and acidified. The *acid* separated from aqueous ethanol as a yellow microcrystalline powder, m.p. 225–226°. (Found: C, 57·4; H, 5·0; equiv., 125. $C_{12}H_{12}O_6$ requires C, 57·1; H, 4·8%; equiv., 126.)

α-(5-Methyl-4-carboxyfurfurylidene)-γ-methyl-$\Delta^{\beta:\gamma}$-butenolide. The above acid (0·5 g.) was refluxed with acetic anhydride (3 ml.) during 4 hr. The mixture was poured into ice water and the brown solid product crystallized several times from methanol. Small yellow needles, m.p. 219–220°. (Found: C, 61·5; H, 4·1. $C_{12}H_{10}O_5$ requires C, 61·5; H, 4·3%.)

α-Hydroxy-β-carbethoxy-γ-phenyl-$\Delta^{\alpha:\beta}$-butenolide. Ethyl sodio-oxaloacetate (4·36 g.; 1 mol.) was ground to a paste with ethanol (7 ml.) and water (20 ml.), the mixture cooled in ice and a solution of freshly distilled benzaldehyde (2·12 g.; 1 mol.) in ethanol (5 ml.) added. After 30 min. the reaction mixture was removed from the ice bath and left overnight at room temperature. Acidification with HCl gave an oil which was taken up in ether, the extract freed from any unchanged benzaldehyde with $NaHSO_3$, washed and evaporated. The solid residue crystallized from aqueous methanol in colourless needles, m.p. 104–105°. (Found: C, 62·7; H, 4·8. $C_{13}H_{14}O_5$ requires C, 62·9; H, 4·8%.) The compound gave a positive reaction with $FeCl_3$, but had no ketonic properties.

SUMMARY

A number of substituted lactones and laevulinic acid derivatives containing furan nuclei have been synthesized. None of the compounds showed any activity when tested as hatching agents for the potato eelworm.

The authors' thanks are due to the Agricultural Research Council and Imperial Chemical Industries Ltd. for their support of this investigation.

REFERENCES

Calam, C. T., Raistrick, H. & Todd, A. R. (1949). *Biochem. J.* **45**, 513.

Calam, C. T., Todd, A. R. & Waring, W. S. (1949). *Biochem. J.* **45**, 520.

Gault, H. & Durand, R. (1943). *C.R. Acad. Sci., Paris*, **216**, 848.

Gonzalez, F. G. (1934). *An. Soc. esp. Fis. Quim.* **32**, 815.

Jones, J. K. N. (1945). *J. chem. Soc.* p. 116.

Kehrer, E. A. & Kleberg, W. (1893). *Ber. dtsch. chem. Ges.* **26**, 345.

Müller, A. & Varga, I. (1939). *Ber. dtsch. chem. Ges.* **72**, 1993.

West, E. S. (1925). *J. biol. Chem.* **66**, 63.

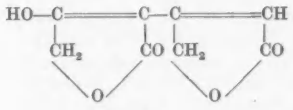
The Potato Eelworm Hatching Factor

6. ATTEMPTS TO PREPARE ARTIFICIAL HATCHING AGENTS. PART II. SOME ACTIVE ARYLIDENE-$\Delta^{\beta:\gamma}$-BUTENOLIDES AND RELATED COMPOUNDS

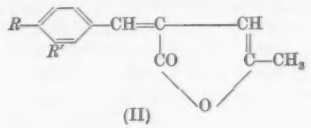
By D. H. MARRIAN, P. B. RUSSELL and A. R. TODD
Chemical Laboratory, University of Cambridge

(*Received* 8 *April* 1949)

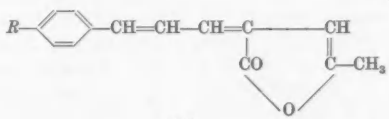
In a previous paper (Russell, Todd & Waring, 1949) the synthesis of a number of furan derivatives for examination as possible hatching agents for cysts of the potato eelworm (*Heterodera rostochiensis* Wollenweber) was reported. None of these materials showed any activity; indeed, out of a large number of substances, both synthetic and natural, belonging to a wide variety of chemical types, only anhydrotetronic acid (I) showed any appreciable activity (Calam, Todd & Waring, 1949). In the course of establishing the structure of anhydrotetronic acid (Marrian, Russell, Todd & Waring, 1947) it was shown that γ-substituted tetronic acids do not yield substituted anhydrotetronic acids, so that the more obvious homologues and analogues of (I) are not readily obtainable. In these circumstances the search for synthetic hatching agents was directed to the preparation of rather different types of compounds, the molecules of which, however, contained conjugated systems analogous to that present in anhydrotetronic acid, e.g. compounds based on α-arylidene-$\Delta^{\beta:\gamma}$-butenolides. The present memoir describes some of this work which has led to the discovery of several biologically active compounds.



(I)



(II)



(III)

α-(*p*-Methoxybenzylidene)-γ-methyl-$\Delta^{\beta:\gamma}$-butenolide (II, R = OMe, R' = H) was first prepared by Thiele, Tischbein & Lössow (1901) by condensation of anisaldehyde with $\Delta^{\beta:\gamma}$-angelicalactone in the presence of a base. Condensations of this type were also carried out by von Oettingen (1930), who prepared a number of compounds including (II, R = OH, R' = OMe), (II, $R = R'$ = OMe) and (II, R and R' being replaced by a methylenedioxy-group). As previously reported, the same general method has been utilized for the preparation of unsaturated lactones containing furan nuclei (Russell *et al.* 1949).

Condensation of *p*-hydroxybenzaldehyde with $\Delta^{\beta:\gamma}$-angelicalactone in the absence of solvent but in presence of a little piperidine, triethylamine or methylmorpholine, gave α-(p-*hydroxybenzylidene*)-γ-*methyl*-$\Delta^{\beta:\gamma}$-*butenolide* (II, R = OH, R' = H). With *m*-hydroxybenzaldehyde the reaction had to be carried out in ethanol as solvent, when α-(m-*hydroxybenzylidene*)-γ-*methyl*-$\Delta^{\beta:\gamma}$-*butenolide* (II, R = H, R' = OH) resulted; under other conditions only a resinous product was formed. Protocatechuic aldehyde gave α-(3:4-*dihydroxybenzylidene*)-γ-*methyl*-$\Delta^{\beta:\gamma}$-*butenolide* (II, $R = R'$ = OH). Condensation of *o*-hydroxyaldehydes did not give compounds of type II, but gave 3-acetonylcoumarins (Marrian & Russell, 1946). The preparation of *o*-hydroxyarylidenebutenolides corresponding to (II) was attempted without success by other methods, e.g. preparation of a butenolide in which the *o*-hydroxy group was blocked by an acid-labile group such as methoxymethyl, followed by acid hydrolysis, failed, although the product obtained by condensation of *p*-methoxymethoxybenzaldehyde with $\Delta^{\beta:\gamma}$-angelicalactone gave an excellent yield of (II, R = OH, R' = H) on acid hydrolysis.

p-Acetamidobenzaldehyde and $\Delta^{\beta:\gamma}$-angelicalactone in the presence of base, with or without solvent, gave α-(p-*acetamidobenzylidene*)-γ-*methyl*-$\Delta^{\beta:\gamma}$-*butenolide* (II, R = NHAc; R' = H). *p*-Aminobenzaldehyde

in ethanol gave under the same conditions α-(p-*aminobenzylidene*) - γ - *methyl* - $\Delta^{\beta:\gamma}$- *butenolide* (II, $R=NH_2$, $R'=H$); the constitution of this compound was proved by acetylation with acetic anhydride, which gave (II, $R=NHAc$, $R'=H$), identical with the sample prepared as above. With succinic anhydride (II, $R=NH_2$, $R'=H$) gave α-(p-*succinamidobenzylidene*) - γ - *methyl* - $\Delta^{\beta:\gamma}$- *butenolide* (II, $R=$-$NHCO(CH_2)_2COOH$, $R'=H$) and with benzene sulphonyl chloride α-(p-*benzenesulphonamidobenzylidene*)-γ-*methyl*-$\Delta^{\beta:\gamma}$-*butenolide* (II, $R=PhSO_2NH$, $R'=H$) was formed. Two cinnamylidene lactones were also prepared. Despite reports to the contrary (von Oettingen, 1930), cinnamaldehyde condensed with $\Delta^{\beta:\gamma}$-angelicalactone to give α-*cinnamylidene*-γ-*methyl*-$\Delta^{\beta:\gamma}$-*butenolide* (III, $R=H$). *p*-Hydroxycinnamaldehyde (Pauly & Wäscher, 1923) did not appear to condense, but p-*acetamidocinnamaldehyde*, prepared by condensation of acetaldehyde with *p*-acetamidobenzaldehyde in presence of potassium hydroxide, gave α-(p-*acetamidocinnamylidene*)-γ-*methyl*-$\Delta^{\beta:\gamma}$-*butenolide* (III, $R=$-NHAc).

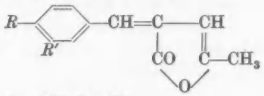
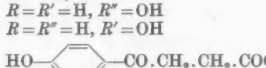
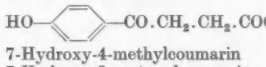
Other compounds prepared for testing were (II, $R=OH$, $R'=OMe$) (von Oettingen, 1930), *p*-hydroxybenzylideneacetone (Zincke & Mulhausen, 1903), *o*-hydroxybenzylideneacetone (Harries, 1891), m-*hydroxybenzylideneacetone*, 3-*o*-hydroxybenzylidene-2:4-diketochroman (Sullivan, Huebner, Stahmann, & Link, 1943), β-(*p*-hydroxybenzoyl)-propionic acid (Swain, Todd & Waring, 1944), methyl-*p*-hydroxycinnamate (Fischer & Nouri, 1917), the amide of *p*-hydroxycinnamic acid, 7-hydroxy-4-methylcoumarin (von Pechmann & Cohen, 1884) and 7-hydroxy-3-acetonylcoumarin (Marrian & Russell, 1946). Attempts were also made to condense 1-acetoxy-4-formylbutadiene-1:4 (glutacondialdehyde-enolacetate), 4-hydroxy-1-naphthaldehyde and indole-3-aldehyde with $\Delta^{\beta:\gamma}$-angelicalactone, but no identifiable product was obtained in any of these cases.

Most of the above compounds were tested as hatching agents for potato eelworm cysts, using the technique already described (Calam, Raistrick & Todd, 1949). The results are shown in Table 1.

In view of the weak activity shown by certain compounds of type (II) it seemed of interest to prepare and test a number of analogously constituted oxazolone derivatives of general formula (IV). For the preparation of such compounds the well-established oxazolone synthesis from aromatic aldehydes and *N*-acylglycines was employed. *p*-Acetamido-benzaldehyde and hippuric acid gave 2-*phenyl*-4-(p-*acetamidobenzylidene*)-*oxazolone*-5 (IV, $R=$—NHAc, $R'=Ph$); with *N*-caproylglycine, 2-n-*amyl* - 4 - (p - *acetamidobenzylidene*) - *oxazolone* - 5 (IV $R=$—$NHCOCH_3$, $R'=n$-C_5H_{11}) together with α-(*N-caproylamino*) - p - *acetamidocinnamic acid* (V, $R=$—NHAc, $R'=$—COC_5H_{11}) resulted. Condensation of *p*-acetamidobenzaldehyde with *N*-acetylglycine gave an unidentified red *acid* $C_{13}H_{16}O_5N_2$, m.p. 246–7°.

Table 1. *Activity of synthetic substances as hatching agents for the potato eelworm*

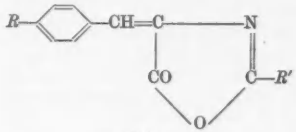
(++, active at concentrations, about 1/5000 to 1/10000; +, active at concentrations, about 1/1000 to 1/5000; ±, active at concentrations, > 1/5000 but variable hatches obtained; −, inactive.)

Substance	Activity
Anhydrotetronic acid	+
	
$R=OH$, $R'=H$	++
$R=NHAc$, $R'=H$	+
$R=NH_2$, $R'=H$	−
$R=OH$, $R'=OMe$	++
$R=H$, $R'=OH$	−
$R=R'=OH$	±
	
$R=OH$, $R'=R''=H$	±
$R=R'=H$, $R''=OH$	−
$R=R''=H$, $R'=OH$	−
	±
7-Hydroxy-4-methylcoumarin	−
7-Hydroxy-3-acetonylcoumarin	−
Methyl *p*-hydroxycinnamate	
Amide of *p*-hydroxycinnamic acid	−
* 3-(*o*-Hydroxybenzylidene)-2:4-diketo-chroman	−
* α-(*p*-Acetamidocinnamylidene)-γ-methyl-$\Delta^{\beta:\gamma}$-butenolide	−

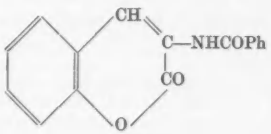
* Very low solubility.

Condensation of the methoxymethyl ether of *p*-hydroxybenzaldehyde with hippuric acid gave 2 - *phenyl* - 4 - (p - *methoxymethoxybenzylidene*)-*oxazolone*-5 (IV, $R=$—OCH_2OMe, $R'=Ph$), which was hydrolysed by a little sulphuric acid in 20 % aqueous acetic acid to α-benzamido-*p*-hydroxycinnamic acid (V, $R=OH$, $R'=COPh$), apparently identical with Erlenmeyer & Halsey's (1899) preparation. Sublimation of this material *in vacuo* at 200° gave 2-*phenyl*-4-(p-*hydroxybenzylidene*) - *oxazolone* - 5 (IV, $R=OH$, $R'=Ph$); a second route to this compound was the hydrolysis of (IV, $R=OCH_2OMe$, $R'=Ph$) with a small amount of H_2SO_4 in glacial acetic acid. The methoxymethyl ether of salicylaldehyde gave 2-*phenyl*-4-(o-*methoxymethoxybenzylidene*)-*oxazolone*-5 with hippuric acid. On hydrolysis with concentrated H_2SO_4 in 20 % aqueous acetic acid, the compound gave 3-benzamidocoumarin (VI), identical with a specimen prepared by the method of Erlenmeyer & Stadlin (1904). Condensation of these methoxymethoxybenzaldehydes with *N*-acetylgly-

cine gave no identifiable product, although condensation of anisaldehyde and *N*-acetylglycine gave the expected product (IV, $R = OMe$, $R' = CH_3$; cf. Sugasawa & Tsuda, 1935). Compounds (IV, $R =$ —NHAc, $R' = Ph$) and (IV, $R =$ —NHAc, $R' = n\text{-}C_5H_{11}$) were tested, but were very insoluble and appeared to be inactive. (IV, $R = OH$, $R' = Ph$) was again very insoluble (1/10,000) in water and showed no activity at this great dilution.



(IV)

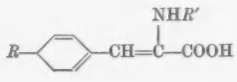


(VI)

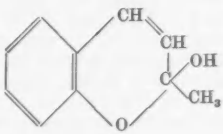
The biological test results recorded in Table 1 show that the *p*-hydroxy compounds (II, $R = OH$, $R' = H$), (II, $R = OH$, $R' = OMe$) and (II, $R = R' = OH$) show definite if weak activity as hatching agents. It is noteworthy that the corresponding *m*-hydroxy compound (II, $R = H$, $R' = OH$) is quite inactive. *p*-Hydroxybenzylideneacetone and β-(*p*-hydroxybenzoyl)-propionic acid also show some activity, while *p*-hydroxyacetophenone, which was also tested, showed a doubtful activity. The *p*-amino compound (II, $R = NH_2$, $R' = H$) was inactive, but replacement of the amino group by the feebly acidic acetamido group as in (II, $R =$ —NHAc; $R' = H$) gave an active substance. Extremely low solubility made the testing of (II, $R = NHCO(CH_2)_2COOH$, $R' = H$) and (II, $R = NHSO_2Ph$, $R' = H$) impossible. Examination of the structure of the active compounds shows that they contain in common with anhydrotetronic acid a weakly acidic group joined through a conjugated system to a $>C{=}O$ group, i.e. HO—(C=C)$_n$—CO— or RCONH—(C=C)$_n$—CO—. Such systems when ionized would exist as resonance hybrids, the negative charge making its appearance at both ends of the system—a state of affairs conducive to hydrogen bonding, which may well play a part in attachment of the hatching agent to some specific protein in the eelworm cysts. Whilst this may be a factor in determining whether a compound can display activity, it is evidently not by any means the only one, as can be seen from the comparatively enormous activity of the natural material and its high specificity. Moreover, the lack of activity in the derivatives of *p*-hydroxycinnamic acid and in the oxazolones examined is evidence pointing in the same direction, since these compounds appear to satisfy the requirements as far as the conjugated system goes. The inactivity of *o*-hydroxybenzylideneacetone, which contains the system

HO—(C=C)$_2$—CO—,

although at first sight surprising, may be due to the



(V)



(VII)

fact that this compound normally exists in the ψ-base form VII (Decker & von Fellenberg, 1909).

In passing, it may be observed that the degree of activity of the compounds may be expressed in a slightly different way from that given in Table 1; anhydrotetronic acid exhibits full hatching activity in water at a dilution of about 1/2000 and α-(*p*-hydroxybenzylidene)-γ-methyl-$\Delta^{\beta:\gamma}$-butenolide (II, $R = OH$, $R' = H$) at a dilution about 1/7500. These activities are much too low for the compounds to have any practical value (purified concentrates of the natural hatching factor show equal activity at dilutions of $1/10^7$), but in view of the high specificity we consider that the results are significant and can form a basis for further synthetic work.

EXPERIMENTAL

α - (p - *Hydroxybenzylidene*) - γ - *methyl* - $\Delta^{\beta:\gamma}$- *butenolide*. *p*-Hydroxybenzaldehyde (1·2 g., 1 mol.) and $\Delta^{\beta:\gamma}$-angelicalactone (0·98 g., 1 mol.) were mixed together and warmed until liquid. Piperidine (2–3 drops) was added to the mixture and the whole warmed on a steam bath for 15 min. More piperidine (3 drops) was added and the mixture allowed to stand overnight, then dissolved in ether and the solution washed with dilute HCl, water, and dried over Na_2SO_4. The ether was removed and the product recrystallized from benzene giving deep yellow needles, m.p. 133–134°. (Found: C, 71·1; H, 5·3. $C_{12}H_{10}O_3$ requires C, 71·4; H, 5·0%.) The substance gives a brick-red colour with NaOH, no colour with $FeCl_3$ and no Legal reaction.

α - (m - *Hydroxybenzylidene*) - γ - *methyl* - $\Delta^{\beta:\gamma}$ - *butenolide*. *m*-Hydroxybenzaldehyde (1·2 g., 1 mol.) and $\Delta^{\beta:\gamma}$-angelicalactone (0·98 g., 1 mol.) were dissolved in absolute

ethanol (10 ml.) and triethylamine (5–10 drops) added. The mixture was refluxed for 5 hr., the solution cooled and water added until it became opalescent. On allowing to stand crystalline material separated, which on recrystallization from aqueous ethanol gave pale yellow needles, m.p. 134°. (Found: C, 70·9; H, 5·0. $C_{12}H_{10}O_3$ requires C, 71·3; H, 4·9%.)

α - (3:4 - *Dihydroxybenzylidene*) - γ - *methyl* - $\Delta^{\beta:\gamma}$- *butenolide*. Protocatechuic aldehyde (2·8 g.) and $\Delta^{\beta:\gamma}$-angelicalactone (1·96 g.) were mixed with triethylamine (5 drops), the mixture warmed for 30 min. on a steam bath and allowed to stand overnight. The mixture was dissolved in ether and the ethereal solution washed with water and dilute HCl, and finally washed free from acid with water and then dried. The ether was evaporated and the residual yellow solid recrystallized from benzene. The product had no definite crystalline form even under the microscope, but repeated recrystallization from benzene left the melting point unchanged at 135°; analysis indicated that it was probably a complex of the desired product with starting material. (Found: C, 62·9; H, 5·0. $C_{12}H_{10}O_4.2C_7H_6O_3$ requires C, 63·1; H, 4·5%.) This material, after several recrystallizations from water, gave the expected lactone as bright yellow needles, m.p. 186°. (Found: C, 65·9; H, 4·8. $C_{12}H_{10}O_4$ requires C, 66·0; H, 4·6%.)

Attempts to prepare α-(o-*hydroxybenzylidene*)-γ-*methyl*-$\Delta^{\beta:\gamma}$-*butenolide*. While condensation of the methoxymethyl ether of *p*-hydroxybenzaldehyde with $\Delta^{\beta:\gamma}$-angelicalactone under the above conditions gave an oil, which on hydrolysis with dilute H_2SO_4 in 50% acetic acid gave formaldehyde and α - (*p* - hydroxybenzylidene) - γ - methyl - $\Delta^{\beta:\gamma}$- butenolide, m.p. 134°, the condensation product from the methoxymethyl ether of salicylaldehyde and $\Delta^{\beta:\gamma}$-angelicalactone gave, on acid hydrolysis under the same conditions, a resinous product from which no pure compound could be isolated.

α - (p - *Acetamidobenzylidene*) - γ - *methyl* - $\Delta^{\beta:\gamma}$- *butenolide*. *p*-Acetamidobenzaldehyde (1·65 g.; 1 mol.) was mixed with $\Delta^{\beta:\gamma}$-angelicalactone (0·98 g., 1 mol.) and piperidine (5 drops) added. The mixture was warmed for 1 hr., allowed to stand for 3 hr., and then triturated with ether and the solid recrystallized from methanol. It gave bright yellow needles, m.p. 196–197° (sintered 185°). (Found: C, 69·5; H, 5·8; N, 6·0. $C_{14}H_{13}O_3N$ requires C, 69·3; H, 5·4; N, 5·8%.) The condensation could be carried out just as effectively in absolute ethanol (25 ml. for above quantities) using triethylamine as catalyst.

α - (p - *Aminobenzylidene*) - γ - *methyl* - $\Delta^{\beta:\gamma}$- *butenolide*. *p*-Aminobenzaldehyde (1·2 g., 1 mol.) and $\Delta^{\beta:\gamma}$-angelicalactone (0·98 g., 1 mol.) were refluxed in ethanol (10 ml.) with triethylamine (6 drops) for 3 hr. The solution was diluted with water until it became opalescent, on cooling orange needles separated, m.p. 138–139°, after recrystallization from methanol. (Found: C, 71·4; H, 5·6; N, 7·1. $C_{12}H_{11}O_2N$ requires C, 71·6; H, 5·5; N, 7·0%.) Treatment with acetic anhydride gave α-(*p*-acetamidobenzylidene)-γ-methyl-$\Delta^{\beta:\gamma}$-butenolide, identical with a sample prepared as above.

α - (p - *Succinamidobenzylidene*) - γ - *methyl* - $\Delta^{\beta:\gamma}$- *butenolide*. α - (*p* - Aminobenzylidene) - γ - methyl - $\Delta^{\beta:\gamma}$- butenolide (1·0 g.) was well mixed with succinic anhydride (0·5 g.), the mixture warmed on a steam bath for 0·5 hr. and then allowed to stand overnight at room temperature. The solid was extracted with saturated $NaHCO_3$ solution, the insoluble portion filtered off and the $NaHCO_3$ solution acidified. The precipitate was collected and washed with water; recrystallized from aqueous ethanol it gave brownish orange needles, m.p. 195–196°. (Found: C, 63·4; H, 4·8; N, 4·7. $C_{16}H_{15}O_5N$ requires C, 63·7; H, 5·0; N, 4·7%.) The $NaHCO_3$-insoluble portion was found to be unchanged α-(*p*-aminobenzylidene)-γ-methyl-$\Delta^{\beta:\gamma}$-butenolide.

α - (p - *Benzenesulphonamidobenzylidene*) - γ - methyl - $\Delta^{\beta:\gamma}$- *butenolide*. α - (*p* - aminobenzylidene) - γ - methyl - $\Delta^{\beta:\gamma}$- butenolide (1·0 g.), benzenesulphonyl chloride (0·8 ml.) and pyridine (5 ml.) were warmed on a steam bath for 30 min. The mixture was poured on ice (30 g.), the solid washed with $NaHCO_3$ solution and recrystallized from ethanol giving yellow needles, m.p. 200–201°. (Found: C, 63·1; H, 4·4; N, 4·2. $C_{18}H_{15}O_4NS$ requires C, 63·4; H, 4·4; N, 4·1%.)

α-*Cinnamylidene*-γ-*methyl*-$\Delta^{\beta:\gamma}$-*butenolide*. Cinnamaldehyde (1·3 g.) and $\Delta^{\beta:\gamma}$-angelicalactone (0·98 g.) were mixed together with triethylamine (3–4 drops) heated for 1 hr. and then allowed to stand overnight. The solid which separated was filtered off and washed with ether; recrystallized from ethanol it gave orange yellow needles, m.p. 139–140°. Found: C, 79·0; H, 5·8. $C_{14}H_{12}O_2$ requires C, 78·8; H, 5·7%.)

p-*Acetamidocinnamaldehyde*. A solution of acetaldehyde (0·4 ml.) in ethanol (15 ml.) was slowly added to an ice-cold stirred solution of *p*-acetamidobenzaldehyde (1·0 g.), and ethanolic KOH (30 ml., 2%). Stirring was continued at 0° for a further 3 hr., the mixture exactly neutralized, evaporated *in vacuo* and the residue extracted thoroughly with ether. The extract was evaporated and the residual red oil dissolved in water (120 ml., with charcoal), filtered and set aside overnight. The solid which separated was recrystallized several times from water giving deep yellow needles, m.p. 175–176° (decomp.). Found: C, 69·7; H, 5·8; N, 7·7. $C_{11}H_{11}O_2N$ requires C, 69·8; H, 5·8; N, 7·4%.) The substance gave a *semicarbazone*, m.p. 229° (decomp.). (Found: N, 22·3. $C_{12}H_{14}O_2N_4$ requires N, 22·8%.)

α - (p - *Acetamidocinnamylidene*) - γ - *methyl* - $\Delta^{\beta:\gamma}$- *butenolide*. *p*-Acetamidocinnamaldehyde (0·65 g.) and $\Delta^{\beta:\gamma}$-angelicalactone (0·31 g.) were mixed together and triethylamine (3–4 drops) added. The mixture was heated on a steam bath for 15 min. and allowed to stand overnight at room temperature. The semi-crystalline mass which separated was triturated with methanol giving orange prisms, m.p. 203–206°. On recrystallization from the same solvent it had m.p. 204–205° (softens 195°). (Found: C, 71·7; H, 5·4; N, 5·2. $C_{16}H_{15}O_3N$ requires C, 71·4; H, 5·6; N, 5·2%.)

m-*Hydroxybenzylideneacetone*. *m*-Hydroxybenzaldehyde (5 g.), acetone (20 ml.) and KOH (14 ml. of 10%) were left to stand at room temperature for 3 days. The solution was acidified with HCl and the oil extracted with ether. After washing with water and drying, the ether was evaporated and the residue recrystallized from benzene giving yellow prisms, m.p. 95–96°. (Found: C, 74·3; H, 6·3. $C_{10}H_{10}O_2$ requires C, 74·1; H, 6·2%.)

2 - *Phenyl* - 4 - (p - *acetamidobenzylidene*) - *oxazolone* - 5. *p*-Acetamidobenzaldehyde (3·26 g.), hippuric acid (3·58 g.), anhydrous sodium acetate (1·68 g.) and acetic anhydride (5·1 g.) were heated together on a steam bath for 45 min. The mixture was cooled and the orange solid triturated with ethanol, filtered and recrystallized from acetic acid; yellow prisms, m.p. 237–239°. (Found: C, 70·4; H, 4·7; N, 9·2. $C_{18}H_{14}O_3N_2$ requires C, 70·6; H, 4·6; N, 9·2%.)

2 - n - *Amyl* - 4 - (p - *acetamidobenzylidene*) - *oxazolone* - 5. *N*-Caproylglycine (3·0 g.) and *p*-acetamidobenzaldehyde

(3·0 g.) were condensed as described above and the resulting solid on recrystallization from methanol gave deep yellow prisms, m.p. 141–142°. (Found: C, 68·2; H, 6·3; N, 9·4. $C_{17}H_{20}O_3N$ requires C, 68·0; H, 6·6; N, 9·3%.) The mother liquors on dilution with water gave a substance which crystallized in pale yellow needles; after recrystallization from aqueous methanol it had m.p. 225–226° (decomp.). (Found: C, 64·5; H, 7·0; N, 9·1. $C_{17}H_{22}O_4N_2$ requires C, 64·3; H, 6·9; N, 9·2%.) This compound was *α-n-caproylamino-p-acetamidocinnamic acid*.

Condensation of acetylglycine with p-*acetamidobenzaldehyde.* Acetylglycine and *p*-acetamidobenzaldehyde were condensed together in the usual manner. The product formed orange-red prisms, m.p. 246–247° from water. After drying *in vacuo* it gave the following analysis. (Found: C, 55·6; H, 5·9; N, 10·0; Equiv., 302. $C_{13}H_{16}O_5N_2$ requires C, 55·7; H, 5·7; N, 10·0%; Equiv., 280.)

2 - *Phenyl* - 4 - (o - *methoxymethoxybenzylidene*)-*oxazolone* - 5. Salicylaldehyde methoxymethyl ether (3·32 g.; Hoering & Baum, 1909), and hippuric acid (3·58 g.) were condensed together in the usual manner. The product crystallized from acetic acid in yellow needles, m.p. 139–140°. (Found: C, 69·6; H, 5·0; N, 4·6. $C_{18}H_{15}O_4N$ requires C, 69·8; H, 4·9; N, 4·5%.)

This material (0·8 g.) was heated on a steam bath for 1·5 hr. with aqueous acetic acid (10 ml., 20%) and H_2SO_4 (4 drops); formaldehyde was evolved. On cooling, colourless needles separated, m.p. 175–176°, undepressed in admixture with 3-*benzamidocoumarin* prepared by the method of Erlenmeyer & Stradlin (1904).

2 - *Phenyl* - 4 - (p - *methoxymethoxybenzylidene*) - *oxazolone* - 5. This was prepared as above from *p*-methoxymethoxybenzaldehyde (Pauly & Wäscher, 1923) and hippuric acid; bright yellow needles from glacial acetic acid, m.p. 122–123°. (Found: C, 70·1; H, 5·1; N, 4·9. $C_{18}H_{15}O_4N$ requires C, 69·8; H, 4·9; N, 4·5%.)

*α-Benzamido-*p-*hydroxycinnamic acid.* The above azlactone (0·5 g.) was hydrolysed with aqueous acetic acid (20 ml., 20%) and H_2SO_4 (4 drops) on a steam bath for 1 hr., by which time no more formaldehyde was evolved. On cooling, colourless prisms separated which on recrystallization from acetic acid had m.p. 227–228° (decomp.), turning yellow at about 200° (Erlenmeyer & Halsey (1899) give m.p. 228–229°).

2-*Phenyl*-4-(p-*hydroxybenzylidene*)-*oxazolone*-5. The above acid was sublimed in a high vacuum at 200° yielding a yellow solid m.p. 220°, undepressed in admixture with a sample prepared by method (ii) below.

(ii) 2 - Phenyl - 4 - (*p* - methoxymethoxybenzylidene) - oxazolone-5 (1·0 g.) was heated to boiling with acetic acid (2 ml.) containing a trace of conc. H_2SO_4. On cooling, a yellow solid separated which, when purified on an alumina column followed by vacuum sublimation at 160°/10^{-6} mm., had m.p. 220–221°. (Found: C, 72·0; H, 4·2; N, 5·2. $C_{16}H_{11}O_3N$ requires C, 72·5; H, 4·2; N, 5·3%.)

SUMMARY

1. A series of compounds has been prepared based on the α-arylidene-$\Delta^{\beta:\gamma}$-butenolide skeleton, and containing conjugated systems analogous to that present in anhydrotetronic acid. Several of the synthetic products show definite, if rather low, activity as hatching agents for the potato eelworm. The structural similarity between the active substances is discussed.

2. No hatching activity was detected in a number of coumarin and oxazolone derivatives.

The authors are indebted to the Agricultural Research Council for grants in aid of this investigation.

REFERENCES

Calam, C. T., Raistrick, H. & Todd, A. R. (1949). *Biochem. J.* **45**, 513.

Calam, C. T., Todd, A. R. & Waring, W. S. (1949). *Biochem. J.* **45**, 520.

Decker, H. & Fellenberg, Th. von (1909). *Liebigs Ann.* **364**, 17.

Erlenmeyer, E. jun. & Halsey, J. T. (1899). *Liebigs Ann.* **307**, 140.

Erlenmeyer, E. jun. & Stadlin, W. (1904). *Liebigs Ann.* **337**, 290.

Fischer, E. & Nouri, O. (1917). *Ber. dtsch. chem. Ges.* **50**, 619.

Harries, C. D. (1891). *Ber. dtsch. chem. Ges.* **24**, 3180.

Hoering, P. & Baum, F. (1909). *Friedl.* **9**, 904.

Marrian, D. H. & Russell, P. B. (1946). *J. chem. Soc.* p. 753.

Marrian, D. H., Russell, P. B., Todd, A. R. & Waring, W. S. (1947). *J. chem. Soc.* p. 1365.

Marrian, D. H., Russell, P. B., Todd, A. R. & Waring, W. S. (1949). *Biochem. J.* **45**, 524.

Oettingen, W. F. von (1930). *J. Amer. chem. Soc.* **52**, 2024.

Pauly, H & Wäscher, K. (1923). *Ber. dtsch. chem. Ges.* **56**, 607.

Pechmann, H. von & Cohen, J. B. (1884). *Ber. dtsch. chem. Ges.* **17**, 2188.

Russell, P. B. Todd, A. R. & Waring, W. S. (1949). *Biochem. J.* **45**, 530.

Sugasawa, S. & Tsuda, T. (1935). *J. pharm. Soc. Japan*, **55**, 1981.

Sullivan, W. R., Huebner, C. F., Stahmann, M. A. & Link, K. P. (1943). *J. Amer. chem. Soc.* **65**, 2288.

Swain, G., Todd, A. R. & Waring, W. S. (1944). *J. chem. Soc.* 548.

Thiele, J., Tischbein, R. & Lössow, E. (1901). *Liebigs Ann.* **319**, 185.

Zincke, Th. & Mulhausen, G. (1903). *Ber. dtsch. chem. Ges.* **36**, 134.

The Fermentation Process in Tea Manufacture

10. THE CONDENSATION OF CATECHINS AND ITS RELATION TO THE CHEMICAL CHANGES IN FERMENTATION

By E. A. H. ROBERTS
Tocklai Experimental Station, Indian Tea Association, Cinnamara, Assam, India

(*Received* 11 *April* 1949)

In an earlier communication in this series, Harrison & Roberts (1939) outlined what was known, at the time, of the chemistry of the so-called tea tannins. More recently Bradfield (1946) and Bradfield, Penney & Wright (1947) have examined the polyphenols in tea by the method of partition chromatography and have established that all the components investigated are derived either from epicatechin or gallocatechin. In view of this positive identification it seems advantageous to refer in future to the polyphenols in tea leaf as catechins.

The reasons for believing that catechins undergo condensation during fermentation after oxidation were summarized by Harrison & Roberts (1939), and further evidence for this belief was afforded by a comparison of the Löwenthal and alkaline iodine titration methods with the Stamm procedure, as applied to tea catechins by Barua & Roberts (1940). Reference to the work of Lamb & Sreerangachar (1940) and Bradfield & Penney (1944) will show that other workers share this view.

The present communication describes experiments, carried out mainly in 1940 and 1941, in which an attempt was made to devise some means of estimating the extent of condensation and to relate it to other chemical changes in fermented tea leaf. A brief outline of these findings has already been presented (Roberts, 1942).

Teas were fermented at temperatures of 60, 70, 80 and 90° F., varying the time of fermentation from 2 to 5·75 hr. Water-soluble solids, water-soluble catechins and alcohol-soluble (i.e. ethanol-soluble) solids were determined on these teas, and the changes in these figures, due to different extents of fermentation, related to the degree of condensation. Less extensive data relating the amount of extractable caffeine to the degree of fermentation are also presented.

Correlations were also found between the extent of condensation and the development of certain liquor characters recognized by the tea taster. These findings will be reported elsewhere.

METHODS

Manufacture of teas

Under normal conditions of manufacture the tea leaf undergoes mechanical rolling for some considerable time, during which period temperature control cannot be maintained. In order to carry out fermentation as far as possible under controlled conditions the period of rolling was reduced to 30 min., after which the leaf was rapidly passed through a McKercher C.T.C. machine. This machine is sometimes used in commercial factories to bring about further bruising of the coarser pieces of tea leaf after rolling is completed. In our experiments it was used to complete the damage necessary for fermentation within a reasonably short time. The remainder of the fermentation was carried out in air-conditioned cabinets at one of four temperatures, 60, 70, 80, or 90° F.; the relative humidity was 95–100%. After 2, 2·75, 3·5, 4·25, 5 and 5·75 hr. fermentation (taken from the time at which rolling was started) samples of leaf were dried at 180° F.

In this way teas which had received twenty-four different fermentation treatments were prepared. The series was repeated eight times in the manufacturing season of 1940. As only two cabinets were available, only two temperatures of fermentation could be dealt with in 1 day. All teas were manufactured from one block of tea, plucked at weekly intervals, so that 2 separate weeks' pluckings were required to cover all four temperatures of fermentation. Temperatures were randomized, so that in the complete series of manufactures, lasting 16 weeks, no one temperature could receive bias owing to seasonal trends.

In all, therefore, 192 different teas were available for investigation, but it was usual to obtain highly significant results with fewer samples.

Analytical procedures

The condensation index. The exact determination of the extent of condensation of catechins is hampered by lack of knowledge, and measurements must therefore be largely empirical. According to Harrison & Roberts (1939) the catechins precipitated from a tea infusion by 1% (v/v) H_2SO_4 probably represent the most highly condensed fraction. Advantage has been taken of this observation and the following procedure adopted.

A portion (20 ml.) of an infusion, obtained by refluxing 5 g. tea with 400 ml. water for 1 hr. and making up to

500 ml., is treated with 0·2 ml. conc. H_2SO_4. After standing for 1 hr. the precipitate obtained is centrifuged down, washed once with 1 % (v/v) H_2SO_4 and suspended in water. The suspension is then titrated according to Stamm's procedure (Barua & Roberts, 1940) and the result expressed as ml. 0·1 M-$KMnO_4$/g. of made tea (dry wt.). The titre is a measure of the catechins precipitated by 1 % H_2SO_4 and is referred to subsequently as the condensation index or C.I. The method must be considered purely tentative, justified at present because it gives semi-quantitative and intelligible results.

Water-soluble catechins were determined by the method of Barua & Roberts (1940), which is applicable to the soluble catechins in green-leaf and fermented tea.

Water-insoluble catechins. The spent tea leaf, after extraction to make up the 1 hr. infusion, is extracted a second time for 1 hr. with 500 ml. water. The residue from the second extract is washed with boiling water, dried and weighed. The dried residue (50 mg.) is suspended in 2 ml. water in a Warburg vessel, and 1 ml. 0·2 N-NaOH tipped in after attainment of temperature equilibrium. The total O_2 uptake, obtained after shaking for 2 hr., expressed as μl. O_2/mg. dry wt. of the original tea, is a measure of the water-insoluble catechins. It may be compared with the figure obtained for the 1 hr. infusion similarly expressed as μl. O_2/mg. dry wt. of made tea.

Caffeine. A 1 hr. or 5 min. infusion (200 ml.) is made alkaline with 10 ml. ammonia (sp.gr. 0·880). The solution is then extracted six times with 50 ml. portions of $CHCl_3$, the solvent removed from the united extracts by distillation and the weight of the residue taken as caffeine.

Oxygen uptake during fermentation. To determine the O_2 uptake which has occurred at any stage of fermentation, the partly fermented leaf is very finely minced and its total uptake determined manometrically. Subtraction of this uptake from the total uptake of a sample of the unfermented green leaf gives the uptake up to the time of sampling (cf. Roberts, 1941).

RESULTS

Variation of condensation index with time and temperature of fermentation. The condensation index of all 192 teas was determined. On three occasions, however, as a result of high withers, in which the moisture content of the tea leaf fell in 18 hr. to about 50 %, instead of the normal 65–70 %, the teas fermented rather more slowly. These three sets have been left out in calculating the mean values for the whole season, which are recorded in Table 1. The increases in the condensation index with the time and temperature of fermentation are almost linear.

Table 1. *Effect of time and temperature of fermentation on the condensation index*

(Values are condensation indices.)

Temp. (° F.)	Fermentation time (hr.) 2	2·75	3·5	4·25	5	5·75
60	24·3	28·6	32·7	36·8	40·0	43·3
70	27·3	32·1	37·3	42·6	46·9	51·1
80	27·6	34·6	41·2	47·7	53·4	59·1
90	33·4	40·2	47·8	55·8	60·3	64·8

For comparison, the O_2 uptake at different stages of fermentation is recorded in Table 2. Each figure is a mean of three separate manufactures.

Table 2. *Oxygen consumption during fermentation*

Temp. (° F.)	Fermentation time (hr.) 2	4	5·75
	(Total μl. O_2/mg. dry wt.)		
60	6·8	8·4	9·6
70	7·6	9·5	10·0
80	8·3	10·4	11·0
90	8·2	10·3	10·8

The results indicate a clear-cut distinction between oxidation and condensation of the catechins. Between 2 and 4·25 hr. fermentation at all temperatures the condensation index increases linearly, with some slight falling off from 4·25 to 5·75 hr., whereas the rate of O_2 uptake decreases as fermentation proceeds. The condensation index increases proportionately with temperature, but the increase in rate of O_2 uptake with temperature is not maintained beyond 80° F.

It would appear logical to conclude that although condensation follows oxidation, the subsequent rate of condensation is independent of the rate of oxidation, and is determined largely by time and temperature.

Water-soluble solids and catechins. It has long been known that the water-soluble solids decrease progressively as a result of fermentation. In Table 3 the mean values are given for water-soluble solids, water-soluble catechins and condensation indices for four times and four temperatures of fermentation. Values for five series of manufacture only are given, as the three high-withered teas were omitted.

As with the condensation index, variations in soluble solids and catechin with time and temperature of fermentation are very highly significant. Correlations between any two of these three variables are also found to be significant, but the eighty pairs of data cannot be considered homogeneous, owing to the systematic differences due to time, temperature and date of manufacture. It is therefore necessary to calculate the correlation coefficient (r) for each factor using the analysis of covariance.

The changes in soluble solids and catechins, due to varying time and temperature of fermentation, are significantly correlated with the condensation index, and with each other (Table 4). The only other significant effect is the residual correlation between soluble solids and catechins. As the catechins account for nearly half the total water-soluble solids, it is not unexpected that such a correlation should be found.

Table 3. *Effect of time and temperature of fermentation on soluble solids, soluble catechins and condensation index*

(C.I. = condensation index.)

Time (hr.)	...	2			3·5			4·25			5·75		
Temp. (° F.)		Soluble solids (%)	Catechin (%)	C.I.	Soluble solids (%)	Catechin (%)	C.I.	Soluble solids (%)	Catechin (%)	C.I.	Soluble solids (%)	Catechin (%)	C.I.
60	Mean	43·2	19·2	26·2	42·1	18·4	35·9	41·8	17·6	40·9	41·1	17·7	48·5
	S.E.	0·25	0·56	1·8	0·51	0·37	2·4	0·51	0·40	2·6	0·47	0·34	3·4
70	Mean	42·3	18·5	27·1	41·2	17·9	38·3	39·9	17·4	44·1	39·7	17·2	52·7
	S.E.	0·78	0·46	1·8	0·91	0·62	1·0	0·74	0·49	1·4	0·73	0·35	1·8
80	Mean	42·0	18·0	30·5	39·7	17·1	46·2	38·5	16·7	54·1	38·3	15·8	63·0
	S.E.	0·35	0·71	0·8	0·57	0·62	1·9	0·72	0·81	2·7	0·99	0·60	0·9
90	Mean	41·1	17·1	34·3	39·4	16·0	49·1	39·1	15·7	57·2	38·5	15·1	65·6
	S.E.	0·56	0·55	3·1	0·38	0·53	3·1	0·45	0·70	3·3	0·64	0·40	2·7

Table 4. *Analysis of covariance. Soluble solids, soluble catechins and condensation index* (C.I.)

	Degrees of freedom	Soluble solids and catechins	Soluble solids and C.I.	Catechins and C.I.
Temperature	3	0·903*	−0·906*	−0·978†
Time	3	0·993‡	−0·969†	−0·997‡
Date	4	−0·038	0·167	0·168
Temperature × time	9	0·143	−0·279	−0·176
Temperature × date	12	0·312	−0·254	−0·231
Time × date	12	0·304	0·516	0·298
Residual	35	0·392*	−0·139	−0·194

* $P = 0{\cdot}05\%$. † $P = 0{\cdot}01\%$. ‡ $P = 0{\cdot}001\%$.

The correlation coefficient (r) between all eighty pairs of data for soluble solids and catechins is 0·5649. If the date variance and the interactions with date are eliminated by averaging each set of five figures, we are left with sixteen comparisons, for which r now becomes 0·9093. A straight line relation may be calculated from these figures. (Catechin %) = −9·6106 + 0·6624 (soluble solids %.)

For a fall of 1·0 % in the water-soluble solids there is a corresponding decrease of 0·66 % in the catechins. Thus, although the decreasing solubility in water of the catechins is a major factor in the decrease in water-soluble solids as a result of fermentation, it is probable that other water-soluble substances are also concerned. This could, perhaps, be accounted for by an oxidation of respiratory substrates to CO_2 by the orthoquinones formed on oxidation of the catechins, or by a combination of oxidized and condensed products with water-soluble nitrogenous substances to form an insoluble product.

Water-insoluble catechins. Table 5 gives average figures of oxygen uptake determined in 1939 for water-soluble and insoluble catechins, obtained by the method of alkaline autoxidation, for teas fermented for 2·5 and 4·75 hr. at 60 and 90° F. The averages are of duplicate experiments in good agreement. The fall in water-soluble catechins is accompanied by a distinct increase in water-insoluble catechins.

Table 5. *Alkaline autoxidation of soluble and insoluble catechins in made tea*

	Temp. (° F.)	Fermentation time (hr.) 2·5	4·75
		μl. O_2/mg. dry wt. original tea	
Soluble	60	42·7	41·0
	90	38·9	34·4
Insoluble	60	9·9	11·7
	90	12·2	13·0

Caffeine. Soluble solids and catechins in the 1 hr. infusion both decrease appreciably as a result of fermentation. The fall in soluble solids cannot entirely be accounted for in terms of loss of soluble catechins, and in the previous section it was pointed out that combination of oxidized catechins with soluble nitrogenous substances to form a water-insoluble product was a possibility.

The results in Table 6 show that with increase in the time or temperature of fermentation there is no detectable effect on the caffeine content in either the 1 hr. or 5 min. infusion. These results prove that during fermentation the water-insoluble products are not produced by combination with caffeine. It might also be expected that, as a result of the known combination of catechins with caffeine, there would be an appreciably lowered extractability of caffeine in the taster's 5 min. infusion, but the results in Table 6 disprove this expectation also.

Table 6. *Effect of fermentation on water-soluble caffeine*

Fermentation		Caffeine content (%)		
Time (hr.)	Temp. (° F.)	5 min. infusion	1 hr. infusion	Condensation index
2	60	2·97	4·30	21·0
2·75	60	3·17	4·40	24·0
4·25	60	2·82	4·50	26·5
4·25	60	3·02	4·50	32·0
5·75	60	3·00	4·35	39·0
4·25	70	2·97	4·50	36·5
4·25	80	2·94	4·35	38·0
2	90	2·97	4·30	32·2
2·75	90	2·92	4·40	37·5
4·25	90	2·94	4·55	58·2
4·25	90	3·25	4·60	47·2
5·75	90	2·90	4·30	66·8

Alcohol-soluble solids. As first established in this laboratory in 1930 (unpublished observations), and independently in Indo-China by Castagnol & Doan-ba-Phuong (1940), alcohol extracts much less material than water from fermented tea. According to the latter authors the percentage of alcohol-soluble solids decreased with increase in fermentation time and the amount of soluble matter, removed in a subsequent extraction of the residue with water, increased; estimation of the catechins by the hide-powder method (Association of Official Agricultural Chemists, 1945) showed that, with increasing time of fermentation, the proportion of catechins fell in the alcohol extract, and increased in the subsequent aqueous extract. The hide-powder method has been found in this laboratory (unpublished observations) to give uncertain results with made tea infusions.

Table 7. *Variation in alcohol and water-soluble solids*

(A.S.S. = alcohol-soluble solids (%); W.S.S. = water-soluble solids (%); C.I. = condensation index.)

Fermentation					
Time (hr.)	Temp. (° F.)	A.S.S.	W.S.S.	W.S.S.–A.S.S.	C.I.
2	60	37·5	44·0	6·5	27·5
2	60	37·0	43·3	6·3	25·5
2	60	37·2	42·8	5·6	25·0
5·75	60	33·2	40·6	7·4	48·0
5·75	60	33·1	42·0	8·9	44·5
3·5	70	33·9	42·5	8·6	37·1
5·75	70	30·6	40·8	10·2	52·8
3·5	80	31·1	38·1	7·0	39·3
2	90	37·7	—	—	24·5
2	90	33·1	39·2	6·1	33·5
4·25	90	27·6	39·5	11·9	56·8
5·75	90	25·6	36·9	11·3	66·8
5·75	90	25·7	37·8	12·1	64·8

Table 7 records values obtained in this laboratory for alcohol-soluble solids (A.S.S.), water soluble products (W.S.S.) and condensation index (C.I.). The regression coefficients of A.S.S. on C.I., and (W.S.S.-A.S.S.) on C.I. are highly significant, the values for t_{11} and t_{10} respectively amounting to 11·13 and 6·90, for both of which P is $< 0{\cdot}001$.

The observations of Castagnol & Doan-ba-Phuong (1940) relating to the extractability of catechins by alcohol are also confirmed, as the catechin content of the aqueous extract of the residue, after alcohol extraction, amounted to 3·79 % for a tea fermented 2 hr. at 60° F. and 6·81 % for a tea fermented 5·75 hr. at 90° F.

Scarcity of solvents made it impossible to extend these observations to caffeine at the time. Recently, however, it has been established that with increasing fermentation the extractability of caffeine in alcohol also decreases. In teas fermented for 2 and 5 hr. at 85° F., 0·41 and 0·60 % caffeine respectively (on dry wt.) has been found in the aqueous extracts of the residue left after extraction with alcohol.

DISCUSSION

Condensation of the catechins could account for changes in chemical composition and in liquor characters, which cannot be interpreted in terms of oxidation alone. An increase in the fermentation time from 3 to 4 hr. in one series of factory experiments, increased the oxidation of catechins from 77 to 80 %. The changes in water-soluble solids, in depth of colour of infusion and in the strength of the liquor, as estimated by a tea taster, were however quite incommensurate with this slight increase in oxidation. The effect of the extra fermentation would be to increase the condensation index from about 40 to 50. If this figure is related to the actual degree of condensation in a linear manner, this represents an increase of 25 % in the condensation, as compared with 3–4 % in the oxidation.

Apart from the obvious variations of liquor characters with time and temperature of fermentation, many of the chemical changes observed can be related to the condensation index. There is, in particular, a very close connexion between this value and the total amount of catechin in solution, increasing condensation resulting in decreased water-solubility of the catechins. This may in part be due to the formation of high polymers of catechins insoluble in water, but judging by the increased ability of the catechins to precipitate gelatine as fermentation proceeds, it appears that this decrease must also be partly due to an actual combination of catechin condensation products with leaf protein to form a water-insoluble product.

The presence of water-insoluble polyphenols in fermented tea has been demonstrated by the method of alkaline autoxidation. These insoluble substances increase with fermentation, but can only be estimated in an empirical way.

The existence of highly polymerized water-insoluble catechins, whether combined with protein

or not, accounts in part for the persistence of the brown colour of spent tea leaf after repeated extractions with water. Oxidation products of chlorophyll, according to Sreerangachar (1943), may also be responsible for the development of a brown pigment in the leaf on fermentation, but the suggestion of Popatov (1932) that these pigments are melanins obtained by oxidation of tyrosine cannot be accepted, as not only is the tyrosine content of tea leaf very small, but tyrosine is not oxidized by either tea oxidase or tea oxidase + catechins (unpublished observations).

The figures available for caffeine in the 1 hr. infusion suggest that there can be little or no combination between catechin condensation products and caffeine to form substances insoluble in boiling water. The values for alcohol-soluble solids suggest, however, that there is an increasing degree of combination between catechins and caffeine as the condensation index increases, with formation of products which are soluble in boiling water but not in boiling alcohol.

Infusions of tea in boiling water are initially clear, but on cooling frequently become cloudy owing to the separation of a finely divided precipitate containing both catechins and caffeine (cf. Bradfield & Penney, 1944). It has always been observed in this laboratory that this effect, known as creaming down, increases with both time and temperature of fermentation. This is a further indication that combination between catechins and caffeine increases with the extent of condensation of the former. Such combination does not decrease the extraction of soluble matter by boiling water, but solubility in cold water and alcohol is appreciably reduced.

SUMMARY

1. An empirical method is described for determining the degree of condensation of catechins in fermented teas.

2. The condensation index increases in approximately linear fashion with time and temperature of fermentation; this increase continues at times and temperatures when the total oxygen uptake is no longer increasing.

3. Values for water-soluble solids and water-soluble catechins, in teas receiving varying fermentation, are significantly correlated with each other, and are correlated negatively with the condensation index. About two-thirds of the loss in soluble solids can be accounted for by an insolubilization of catechins, due to the formation of high polymers insoluble in water, and by combination of condensation products with leaf protein.

4. The much greater fall in alcohol-soluble solids with increase in the condensation index indicates a combination of condensation products with water-soluble nitrogenous compounds to form complexes soluble in boiling water, but insoluble in boiling alcohol.

The author wishes to express his thanks to Mr C. J. Harrison, Chief Scientific Officer, for his interest in this work and to the Indian Tea Association for permission to publish these results.

REFERENCES

Association of Official Agricultural Chemists (1945). *Official and Tentative Methods of Analysis*, p. 112. Washington: Association of Official Agricultural Chemists.

Barua, D. N. & Roberts, E. A. H. (1940). *Biochem. J.* **34**, 1524.

Bradfield, A. E. (1946). *Chem. & Ind.* **28**, 242.

Bradfield, A. E. & Penney, M. (1944). *J. Soc. chem. Ind., Lond.*, **63**, 306.

Bradfield, A. E., Penney, M. & Wright, W. B. (1947). *J. chem. Soc.* p. 32.

Castagnol & Doan-ba-Phuong (1940). *Bull. écon. Indochine*, **4**, 645.

Harrison, C. J. & Roberts, E. A. H. (1939). *Biochem. J.* **33**, 1408.

Lamb, J. & Sreerangachar, H. B. (1940). *Biochem. J.* **34**, 1472.

Popatov, A. I. (1932). *The Biochemical Basis of Technology of Tea*, Tiflis.

Roberts, E. A. H. (1941). *Biochem. J.* **35**, 909.

Roberts, E. A. H. (1942). *Advanc. Enzymol.* **2**, 113.

Sreerangachar, H. B. (1943). *Curr. Sci.* **12**, 205.

The Deamination of Glycine by α-Radiation from the Disintegration of Boron in a Nuclear Reactor

By W. M. DALE, J. V. DAVIES and C. W. GILBERT
Christie Hospital and Holt Radium Institute, Manchester

(*Received* 28 *June* 1949)

A correct assessment of the effects of α-radiation as an example of densely ionizing radiations, in comparison with a less densely ionizing radiation (e.g. X-rays), on a variety of solutes in aqueous solutions, is of importance for an understanding of the mode of action. There are a few such comparative investigations reported in the literature (Dale, Gray & Meredith, 1949; this paper contains references to relevant literature). In general α-radiation is less effective than X-radiation. In some of these investigations it is doubtful whether there was a sufficient consideration of the part played by simultaneous β- and γ-radiation and, therefore, the small effect observed may have been entirely due to radiation other than α-rays. Such effects were fully taken into account in a recent investigation on a comparison between α- and X-radiation effects on carboxypeptidase (Dale, Gray & Meredith, 1949). It was also shown that an increase of the concentration of the solute led to an increase of the α-ray effect, and the authors inclined to the view that the effects observed were due to the δ-rays issuing from the α-ray track. Radiation from a polonium source would entirely avoid interference by β- or γ-radiation, but it is experimentally difficult to make sufficiently strong sources of radiation. This paper reports an investigation of the effects of α-radiation using the disintegration particles from the action of slow neutrons on a boron salt present in the solution of the solute to be investigated. The advantage of this procedure is that not only a large radiation dose can easily be obtained using the neutron flux available in nuclear reactors, but also that the yield from the interfering β- and γ-radiation can be directly measured.

The reaction investigated was the deamination of glycine by radiation, and this reaction is of interest for several reasons. Glycine has a low molecular weight (75) in contrast to the high molecular weight of carboxypeptidase (35,000); furthermore, solutions of higher concentrations than are possible with carboxypeptidase can be made, and finally the deamination has been shown (Dale & Davies, 1949; Dale, Davies & Gilbert, 1949) to have an ionic yield for X-rays rising to nearly 3 on increasing the concentration of glycine to 20%, whereas the X-ray ionic yield with carboxypeptidase remains constant at 0·19 over a very wide range of concentrations.

EXPERIMENTAL

Dosimetry

The exposure to slow neutrons of a solution containing a B salt produces an irradiation of the solution by α-particles and slow Li particles resulting from the nuclear reaction ^{10}B $(n\alpha)$ ^{7}Li. With a neutron flux of 10^{10}/cm.^{2}/sec. or more, available in nuclear reactors, one can obtain in a few hours sufficient radiation from the heavy particles to give good yields of NH_3 from the deamination of glycine. However, in a nuclear reactor there is also a considerable flux of fast neutrons and γ-radiation and these will produce further deamination via their corresponding secondary radiations, protons and electrons. There is also the slow neutron reaction ^{14}N (np) ^{14}C originating from the nitrogen in the glycine. These effects can be eliminated by exposing two solutions of the same glycine concentration side by side in the neutron flux, one containing a B salt and one without, the difference in the yields being due to the heavy particle radiation only. We, in fact, irradiated a third solution containing double the amount of B salt, to check that the deamination by the α-particles was proportional to the B concentration. For reliable results the deamination in presence of B should be as large as possible compared with the background effect, and this is achieved by using the highest possible concentration of B. It was found by preliminary experiments that up to 2·25 M-KBO_2 (potassium metaborate) could be added to the glycine solution without affecting the deamination yields by X-radiation. The resulting solution was, however, strongly alkaline, and accordingly special experimental procedure had to be adopted, as discussed later. Little prior information about the intensity of the γ-radiation in the nuclear reactor was available, but rough estimates indicated that this amount of B would be sufficient, as was finally shown by the experiment.

The rate of liberation of energy/unit volume of solution by the B disintegration can be calculated from the B concentration, the disintegration cross-section for thermal neutrons and the average neutron flux in the solution. The mean energy release in the slow neutron disintegration of B can be calculated from the masses (Mattauch & Flügge, 1942), remembering that only about 8% of the disintegrations liberate the full energy, the remainder leaving the ^{7}Li in an excited level of 474 keV. (Bøggild, 1945; Gilbert, 1948; Rosenfeld, 1948). These calculations give a mean energy/disintegration of 2·49 MeV. The effect of the 474 keV. γ-radiation resulting from the de-excitation of the ^{7}Li will not be eliminated by comparison with the B-free solution, but the solution was in a layer of only 2 mm. thickness and absorbs a negligible amount of the γ-radiation. The

capture cross-section for the B disintegration is taken as 703×10^{-24} cm.^{2}, and therefore the rate of energy liberation/ml. of molar solution of B for a neutron flux n/cm.^{2}/sec. is

$$6{\cdot}06 \times 10^{20} \times 703 \times 10^{-24} \times 2{\cdot}49 \times 10^6 \, n = 1{\cdot}06 \times 10^6 \, n \text{ eV./sec.}$$

For the purpose of calculating the ionic yield we can define an ion pair as the absorption of 32·5 eV. and hence the rate of production of ion pairs/ml. of M-borate solution due to the α and Li particles is $3{\cdot}26 \times 10^4$ n/sec.

For the determination of n, the average neutron flux, account must be taken of the considerable local disturbance of the neutron flux produced by the presence of so much B, and also of the fact that the flux will vary markedly throughout the solution. These effects can be allowed for by using the presence of K as a monitor, through the activity of the ^{42}K produced by the slow neutron reaction ^{41}K $(n\gamma)$ ^{42}K, for the number of ^{42}K atoms produced is proportional to the number of B disintegrations independent of the spatial variations of the neutron flux. All solutions were therefore made to have nominally the same K concentration, the appropriate amount of K_2CO_3 being added to the solutions with less or no KBO_2.

Since the half life of ^{42}K is 12·4 hr. and the irradiation of the B solution was 20·9 hr. allowance was made for the decay of the ^{42}K during irradiation in calculating the integrated dose. A similar correction for decay between the time of irradiation and measurement was also made. The activity of a K sample was followed for 72 hr. to check the half life and a value of 12·8 hr. was obtained. This is higher than the generally accepted value of 12·44 (Seaborg & Perlman, 1948) by more than the statistical errors. The measured value was used in the corrections. If this higher value were due to an impurity, its adoption gives the smaller error.

The K monitor was calibrated by two methods. In the first, some samples of 1·48 M-K_2CO_3 solution were irradiated for several days in the nuclear reactor in positions where the neutron flux had been calibrated. The second method involved calibrating the efficiency of a Geiger counter for ^{42}K and using the activation cross-section for ^{42}K (Seren, Friedlander & Turkel, 1947). The counter was of the Veall type (Veall, 1948) and was calibrated with a ^{42}K solution which had been measured at the Atomic Energy Research Establishment, Harwell, in a counter of known efficiency. The two methods give results which differ by about 14%, the first method giving the lower value of the neutron flux. This difference probably indicates the order of magnitude of the error in determining the absolute value of the neutron flux. The mean of the two methods was adopted in the calculations. The neutron flux determined by this method for the B-free solutions compared well with the estimated value for their positions in the nuclear reactor.

Chemical method

The general chemical procedure for estimating the NH_3 liberated in the deamination by radiation was the same as in previous work (Dale, Davies & Gilbert, 1949), but since the highly concentrated solution of KBO_2 required could not be neutralized without precipitation it was necessary to carry out the irradiation in the Conway units themselves to prevent loss of NH_3. The standard pyrex or soda glass Conway units are not suitable for irradiation inside a nuclear reactor because of the boron and sodium content of the glasses, which either absorb neutrons too strongly or are too radioactive after irradiation. Perspex was therefore used, and sets of three units were hollowed out of solid blocks $6 \times 2 \times 1$ in. with perspex sheets ground to form covering lids, which were held in place by clips. Each block was packed into an aluminium can with absorbent cotton wool for insertion into the nuclear reactor. The three Conway units in each block had inner compartments and outer rings which were filled with 1 ml. of fluid to a depth of about 2 mm. An 0·25 N-H_2SO_4 solution was put in all inner compartments. The outer rings contained the glycine solution with varying amounts of KBO_2 and K_2CO_3, the glycine content being the same in the three units of any one block. The same pattern of KBO_2 and K_2CO_3 concentration was repeated in all blocks and is shown in Table 1.

Two glycine concentrations were investigated, 200,000 and 10,000 μg./ml. made up by weight, the higher concentration being duplicated. In all, three perspex blocks were irradiated for 20·9 hr. in a neutron flux of about 10^{10}/cm.^{2}/sec. in the nuclear reactor GLEEP at the Atomic Energy Research Establishment, and after removal from the reactor the units were rocked for 0·5 hr. to ensure the completion of the diffusion of NH_3 into the acid solution. The solutions from all compartments in the Conway dishes were then transferred quantitatively to test tubes and the subsequent measurements made in our laboratory in Manchester.

Blank experiments to determine the yield of NH_3 in the absence of radiation were made at the same temperature (31°) experienced during the irradiation, and for the same duration, and also control experiments under the same conditions but with added H_2O_2. According to Bonet-Maury & Lefort (1948) H_2O_2 is formed in pure water proportionately to the α-ray dose. If a solute is present which is sensitive to H_2O_2 part of the radiation effect might be due to H_2O_2 formed during irradiation. On extrapolating the curves published by these authors to the two doses of α-radiation used in the present experiment, one arrives at H_2O_2 concentrations of $7{\cdot}35 \times 10^{-3}$ and $3{\cdot}68 \times 10^{-3}$ M, which are the average concentrations of H_2O_2 built up during the experiment in the nuclear reactor. These concentrations are maximum concentrations, since one can assume that the presence of a solute reacting with OH radicals and/or H atoms will reduce the formation of H_2O_2.

RESULTS

The results of the α-radiation are summarized in Table 1. The ionic yields of the heavy particle radiation are 0·56 and 0·17 for the 200,000 and the 10,000 μg./ml. solutions respectively. Comparing these with the corresponding yields for X-radiation (Dale, Davies & Gilbert, 1949) the relative efficiencies for deamination by α-radiation and X-radiation were 19·5% for the 20% solution, and 15·2% for 1% solution.

Table 2 shows the ammonia yield due to hydrogen peroxide. The hydrogen peroxide effect, therefore, is at most 12·5% of the α-ray effect for the 200,000 μg./ml. glycine solution and 7·5% for the 10,000 μg./ml. solution.

Table 1. *Deamination of glycine by α-particles*

	Block 1			Block 2			Block 3		
Conway unit	A_1	B_1	C_1	A_2	B_2	C_2	α	β	γ
Glycine concn. (μg./ml.)		200,000			200,000			10,000	
KBO_2 concn. (M)	2·25	0	1·13	2·25	0	1·13	2·25	0	1·13
Total K concn. (M)	2·25	2·96	2·60	2·25	2·96	2·60	2·25	2·96	2·60
NH_3 yield (μg./ml.)	800	73	539	968	65	462	312	26	180
Blank (μg./ml.)	12	3	12	12	3	12	1	1	2
Net NH_3 yield (μg./ml.)	788	70	527	956	62	450	311	25	178
NH_3 yield due to α-rays (μg./ml.)	718	—	457	894	—	388	286	—	153
Mol. NH_3/ml. $\times 10^{-19}$	2·57	—	1·64	3·20	—	1·39	1·02	—	0·55
Potassium activity* $\times 10^{-5}$	1·36	1·64	1·31	1·20	1·35	1·17	1·48	1·74	1·48
Neutron flux $\times 10^{-10}$	1·02	1·24	0·99	0·90	1·02	0·89	1·12	1·31	1·12
Ion pairs/ml. $\times 10^{-19}$	5·64	—	2·76	4·99	—	2·47	6·20	—	3·10
Ionic yield for α-radiation	0·46	—	0·59	0·64	—	0·56	$0{\cdot}16_5$	—	$0{\cdot}17_7$
Mean ionic yield			0·56					$0{\cdot}17_7$	
Ionic yield for X-radiation†			2·89					1·13	
Ratio ('α yield' as % of 'X yield')			19·5%					15·2%	

* Potassium activity is the counts/min./ml./'molar' K, 58 hr. after the end of the irradiation.
† Dale, Davies & Gilbert (1949).

Table 2. *Deamination of glycine by hydrogen peroxide at 31° during 21 hr.*

Glycine concn. (μg./ml.) ...	200,000		10,000	
H_2O_2 concn. (mM)	7·35	3·68	7·35	3·68
NH_3 yield (μg./ml.)	104	67	25	11
Blank (μg./ml.)	12	12	1	1
NH_3 due to H_2O_2 (μg./ml.)	92	55	24	10

DISCUSSION

In Table 3 are set out the ammonia yields from the boron-free solutions, which will be due to radiation by electrons and protons, reduced to a standard neutron flux of 10^{10}/cm.²/sec. On the assumption that the ionic yield for these radiations is the same as for X-radiation, the total dose and the dose rate in r./min. has been calculated. This estimation, however, only gives a lower limit to the radiation dose, because the proton part, having a higher ion density, is probably not so efficient in deamination as the electron part, but there is reasonable agreement between the values calculated from solutions of different concentrations.

Table 3. *Effect of background radiation*

Glycine concn. (μg./ml.)	Block 1 200,000	Block 2 200,000	Block 3 10,000
NH_3 yield/ml. for neutron flux of 10^{10}/cm.²/sec.	57	61	19
Mol. NH_3/ml. $\times 10^{-18}$	0·70	0·75	0·61
Total dose $\times 10^{-5}$ (r.)	4·35	4·66	3·78
Dose rate in flux 10^{10} r./min.	347	372	302

In any comparison between radiation effects produced by the boron disintegration and those by α-particles from radon or polonium account must be taken of the different densities of the ions along the tracks of the particles. The boron disintegration produces two ionizing particles, an α-particle with range in standard air of 0·77 cm. or 7·7 μ. in water and a Li particle of range 0·48 cm. in air or 4·8 μ. in water (Gilbert, 1948). The maximum ion density of the latter is only just greater than that of the α-particle (Bower, Bretscher & Gilbert, 1938) because its range is shorter than the Bragg maximum in the ionization curve for lithium particles. The mean linear density of ionization along the disintegration track is 5900 ion pairs/μ. of track in water, the mean density of the lithium particle being about 13% less than in the α-track. For comparison the corresponding figure for the mean of the three α-particles emitted by radon and its short-lived daughter products is 3500 ion pairs/μ. and for the electrons produced by a 200 kV. X-ray tube 80 ion pairs/μ. (Gray, 1947).

The relative yield for α-particles compared with X-radiation (15–19%) for deamination of glycine is considerably higher than the values (3–10%) for a range of concentrations for the inactivation of carboxypeptidase by α-particles from radon and its short-lived products (Dale, Gray & Meredith, 1949). The increased efficiency in the present experiment is not likely to be due to the fact that the linear ion density was greater than in the radon experiment, for this should rather result in a decreased efficiency. Both experiments show an increase of the ionic yields for α-radiation with concentration of solute, though not proportional to concentration. The high value of 0·56 obtained for the highest concentration of glycine is of particular interest showing that the whole effect cannot be attributed to the δ-rays but

that the main column of the α-track must play an important part.

Bonet-Maury & Lefort (1948) have shown that hydrogen peroxide is formed in pure water by α-radiation, and therefore part of the α-radiation effect could be due to the presence of hydrogen peroxide if the solute is sensitive to it. The presence of a solute however may reduce the formation of hydrogen peroxide by reacting with the primary products of radiations (OH radicals and H atoms). Carboxypeptidase was shown to be insensitive to high concentrations of hydrogen peroxide (Dale, Gray & Meredith, 1949) and though the hydrogen peroxide can deaminate glycine, the effect is not more than 12% of the total radiation effect as shown by the present control experiments.

Since neither the quality of the radiation nor the effect of hydrogen peroxide can wholly account for the observed differences, one should assume that these are linked in some way with the fact that the X-radiation effects on amino-acids were very different from those on macromolecules, as was fully discussed in an earlier paper (Dale, Davies & Gilbert, 1949). There a chain reaction or the action of radicals by excitation was invoked for explaining the kinetics of the deamination of amino-acids by X-rays, and it was also pointed out that these theoretical considerations are not yet fully in agreement with current concepts of the indirect action of radiation.

SUMMARY

1. The deamination of glycine by the densely ionizing particles from the disintegration of boron by slow neutrons in a nuclear reactor has been studied. An account of the necessary technique is given and the dosimetry fully discussed.

2. The ionic yields for the liberation of ammonia were 0·17 and 0·56 for 1 and 20% solutions of glycine, respectively, being 15 and 19% of that due to X-radiation. The higher efficiency of deamination by this radiation compared with the inactivation of an enzyme by α-rays from radon and its products is discussed.

We wish to thank the Director, Atomic Energy Research Establishment, Harwell, for permission to make the irradiation in the nuclear reactor and the staff of the Isotope Division for information concerning the neutron flux, for a standardized solution of ^{42}K and for the assistance and facilities given during the irradiation.

REFERENCES

Bøggild, J. (1945). *Math.-fys. Medd.* **23**, 4.
Bonet-Maury, P. & Lefort, M. (1948). *Nature, Lond.*, **162**, 381.
Bower, J. C., Bretscher, E. & Gilbert, C. W. (1938). *Proc. Camb. phil. Soc.* **34**, 290.
Dale, W. M. & Davies, J. V. (1949). *Nature, Lond.*, **163**, 64.
Dale, W. M., Davies, J. V. & Gilbert, C. W. (1949). *Biochem. J.* **45**, 106.
Dale, W. M., Gray, L. H. & Meredith, W. J. (1949). *Philos. Trans.* A, **242**, 33.
Gilbert, C. W. (1948). *Proc. Camb. phil. Soc.* **44**, 447.
Gray, L. H. (1947). *Brit. J. Radiol.* Suppl. no. 1, p. 7.
Mattauch, J. & Flügge, S. (1942). *Kernphysikalische Tabellen.* Berlin: Springer.
Rosenfeld, L. (1948). *Nuclear Forces.* Amsterdam: North Holland Publishing Co.
Seaborg, G. T. & Perlman, I. (1948). *Rev. mod. Phys.* **20**, 585.
Seren, L., Friedlander, H. N. & Turkel, S. H. (1947). *Phys. Rev.* **72**, 888.
Veall, N. (1948). *Brit. J. Radiol.* **21**, 347.

Observations on Antisera Against Complexes of Di-2-chloroethyl sulphide and Protein

By D. S. FLEMING, A. M. MOORE and G. C. BUTLER
Chemical Warfare Laboratories, Ottawa, the Department of Bacteriology and Immunity, McGill University and the Department of Biochemistry, University of Toronto

(*Received* 31 *January* 1949)

Human beings and animals, after receiving repeated skin exposures to di-2-chloroethyl sulphide (mustard gas, '*H*'), often develop an increased sensitivity to this substance, and it was felt that the factors concerned might be investigated by methods developed in the study of immunity. Berenblum & Wormall (1939) previously published an account of an investigation undertaken with this end in view, and the present communication provides additional information about the system first described by these authors. It was also hoped that by studying different derivatives of diethyl sulphide as inhibitors in a specifically precipitating immunity system, some evidence might be obtained for the nature of the linkage formed when *H* reacts with proteins.

There have appeared in recent years a number of reports describing the action of *H* on proteins, e.g. Banks, Boursnell, Francis, Hopwood & Wormall (1946); Boursnell, Francis & Wormall (1946*b*); Herriott, Anson & Northrop (1946); and Ogston (1948). The serological investigation which forms the subject of the present communication has disclosed another factor in such reactions, viz. the participation of inorganic phosphate.

EXPERIMENTAL

Preparation of compounds

Di-2-(2′-amino-2′-carboxyethylthioethyl) sulphide. Prepared by interaction of *H* with cysteine in the manner described by Hellerman (1942), this had m.p. 245°.*

1:4-Thiazan-4-acetic acid and 2-hydroxy-2′-glycino-diethyl sulphide. Ethyl aminoacetate and *H* were brought into reaction in absolute ethanol in the presence of anhydrous sodium acetate, and the crude oily product was isolated following the method of Cashmore & McCombie (1923). Two fractions were obtained from this product, one with b.p. 91–92°/1 mm., the other with b.p. 100–140°/1 mm. Each of these fractions was dissolved in dry ether, dry HCl gas was bubbled through the solution and the crystalline hydrochloride which separated in each case was collected on the filter with suction and recrystallized from an ethanol ether mixture (2/5).

From 10 g. of the oil (b.p. 91–92°/1 mm.) 10 g. of recrystallized hydrochloride with m.p. 185–187° were obtained. (Found: C, 42·5; H, 7·1; N, 6·2; S, 14·4; Cl, 15·7%. The *hydrochloride* of *ethyl* 1:4-*thiazan-4-acetate*, $C_8H_{16}O_2NClS$, requires C, 42·6; H, 7·2; N, 6·2; S, 14·2; Cl, 15·8%.) This ethyl ester hydrochloride (5 g.) and N-NaOH (100 ml.) were refluxed for 4 hr., after which the mixture was cooled; N-H_2SO_4 (78 ml.) was then added and the solution distilled to dryness *in vacuo*. The residue was extracted three times with boiling ethanol, the extracts filtered and reduced to dryness, and the residue crystallized from ethanol. Yield, 3·0 g. The resulting needle-shaped crystals showed a peculiar behaviour in melting-point determinations. In a capillary tube they melted at 130°; above this temperature some samples decomposed with much evolution of gas, while other samples recrystallized and melted at 175°. On the heated stage of a Fisher-Johns micro melting-point apparatus they had m.p. 175–176° with no preliminary melting at 130°. This transient melting at 130° was probably due to loss of water of crystallization, since analyses showed that the crystals contained water. A sample of the crystals was sublimed at 160°/0·15 mm.; the sublimate melted and turned red at 177–179° without any preliminary melting at 130°. (Found for the sublimate: C, 44·5; H, 7·4; N, 8·5; S, 20·2. Calc. for $C_6H_{11}O_2NS$: C, 44·7; H, 6·9; N, 8·7; S, 19·9%.) There seems little doubt that this material is identical with the 1:4-thiazan-4-acetic acid described by Boursnell, Francis & Wormall (1946*a*).

From 7 g. of the oil (b.p. 100–140°/1 mm.) 2 g. of recrystallized hydrochloride with m.p. 120–122° were obtained. (Found: C, 42·0; H, 6·9; N, 5·1; S, 11·4; Cl, 12·1. Calc. for the ethyl ester hydrochloride of 2-acetoxy-2′-glycino-diethyl sulphide, $C_{10}H_{20}O_4NClS$: C, 42·1; H, 7·05; N, 4·9; S, 11·4; Cl, 12·4%.) This ethyl ester hydrochloride (2 g.) and N-NaOH (30 ml.) were refluxed for 2 hr., after which the mixture was cooled, N-H_2SO_4 (20 ml.) was added and the solution was distilled to dryness *in vacuo*. The residue was extracted three times with boiling ethanol, the extracts filtered and reduced to dryness, and the residue crystallized by dissolving it in 10 ml. of hot ethanol, adding 2 ml. of acetone and chilling. The crystals separated as rosettes of needles melting at 129–131°. The material was very soluble in water, ethanol and methanol, but relatively insoluble in acetone, ether and benzene. (Found: C, 40·3; H, 7·4; N, 7·8; S, 18·0. Calc. for $C_6H_{13}O_3NS$: C, 40·2; H, 7·3; N, 7·8; S, 17·9%.)

The analyses of this material and of the ester hydrochloride from which it was derived provide good, though not conclusive, evidence that it is 2-hydroxy-2′-glycinodiethyl sulphide, and it is quite conceivable that the acetate of the ethyl ester of this compound could have arisen as a result of the reaction of 1 mol. of *H* simultaneously with 1 mol. of sodium acetate and 1 mol. of ethyl aminoacetate.

In the course of several preparations of the sort just described many attempts were made to obtain the di-2-glycinoethyl sulphide described by Cashmore & McCombie (1923), but without success.

H-glycine.† Glycine (10 g.), water (200 ml.) and *H* (30 ml.) were placed in a 500 ml. beaker and stirred continuously at 37°, 2N-NaOH being added at a rate sufficient to keep the reaction alkaline to phenolphthalein. After 8 hr. the consumption of alkali had ceased when 260 ml. of 2N-NaOH had been added. The reaction mixture was then extracted three times with chloroform and the chloroform washings discarded. The aqueous solution was concentrated *in vacuo* on the steam bath to about 100 ml. when a large amount of NaCl had separated; the sludge in the distilling flask was filtered with suction and the solid on the filter washed with a little cold water. The pH of the filtrate was adjusted to about 2 by the addition of conc. HCl. As the acidification proceeded a copious, gummy, slightly yellow precipitate formed and rapidly settled to a gummy mass at the bottom of the flask. After the mixture had been chilled for 2 hr. at 5° the supernatant liquid was decanted and discarded and the gummy precipitate washed twice with cold water. The remaining gum was then dissolved in 100 ml. water by adding 2N-NaOH until the pH of the solution was 6·5. The precipitation with acid, washing and redissolving of the product was carried out twice more; the resulting gum was dissolved in water at pH 6·5 and the solution diluted to 50 ml. This solution had 5·3 mg. N/ml.; yield, 14% (based on N).

A solution which had been made from a similarly prepared and purified reaction product gave: total N (Kjeldahl) 0·93 mg./ml.; amino N (Van Slyke), 0·00 mg./ml.; S, 1·46 mg./ml. When 5 ml. of this solution was mixed with 0·1 ml. of a 10% solution of any one of cobaltous acetate, cadmium chloride, nickel nitrate or cupric sulphate a copious precipitate formed. The chemical properties of this *H*-glycine received considerable study which will be the

* All m.p. are uncorrected.

† The '*H*-glycine' referred to here is not to be confused with the '*H*-glycine' used by Boursnell *et al.* (1946*a*) to mean 1:4-thiazan-4-acetic acid, but refers to the unidentified product of the reaction of *H* and glycine in aqueous solution.

subject of a communication by Drs D. E. Douglas and R. D. H. Heard.

'*Thiodiglycol phosphate.*' $Na_2HPO_4.7H_2O$ (6·7 g.) was dissolved in water (100 ml.) and the solution was maintained at 37°; 4 g. of *H* (m.p. 14°) were then added and the reaction mixture stirred continually. The reaction was kept just alkaline to phenolphthalein by the addition of 2N-NaOH. After 2 hr., when the reaction was over, 19 ml. of 2N-NaOH had been used and an analysis for inorganic phosphate showed that 80% of that originally present had been esterified. The reaction mixture was distilled to dryness *in vacuo* and the residue was washed with absolute ethanol, the washings being discarded. The residue was then dissolved in 5 ml. of water, 50 ml. of absolute ethanol were added and the mixture was chilled at 5° for 3 hr. when the clear supernatant liquid was decanted and discarded. The solution and precipitation were carried out twice more after which the residue was dissolved in water and diluted to 50 ml. This solution gave: P (total), 12·5 mg./ml.; P (inorganic), 3·6 mg./ml.; S, 8·2 mg./ml.

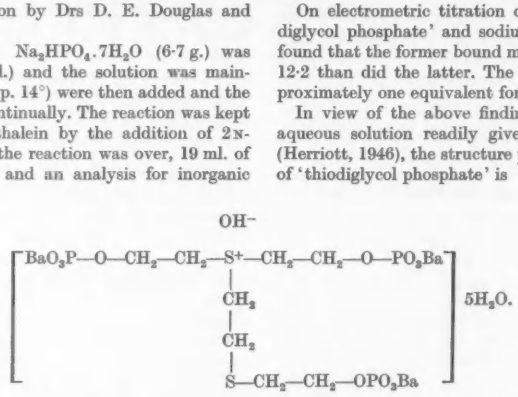
The above solution was made alkaline to phenolphthalein by the dropwise addition of a saturated $Ba(OH)_2$ solution; the precipitate which formed was centrifuged and discarded. To the supernatant fluid $Ba(OH)_2$ dissolved in hot water was added in the proportions of one atom of Ba for each atom of P. The precipitate which resulted was centrifuged and washed three times with small volumes of distilled water. To the combined supernatant and washings, which were perfectly clear and colourless, an equal volume of ethanol was added and the resulting voluminous precipitate centrifuged, washed twice with 50% and twice with 95% ethanol, and dried *in vacuo* over $CaCl_2$. (Found: C, 10·0; H, 2·8; S, 6·3; P, 9·5; Ba, 41·2. $C_2H_{16}O_{13}S_2P_2Ba_2.5H_2O$ requires: C, 9·8; H, 2·7; S, 6·6; P, 9·5; Ba, 42·1%.)

It can be seen that the ratio P/S in this material is 3:2 and at one time it was suspected that this might be due to a mixture of thiodiglycol mono- and di-phosphates. In order to test this possibility a brucine salt was prepared and analysed for P and S. Aqueous solutions of the above Ba salt (0·5 g.) and brucine sulphate (1·5 g.) were mixed, $BaSO_4$ was removed by centrifuging and the resulting solution was evaporated *in vacuo*. Crystallization of the residue from water yielded 0·663 g. of fine white needles. Even after recrystallization this brucine salt had no definite m.p. but softened, melted and decomposed gradually from 110–185°. (Found: S, 2·32; P, 3·35%.) These analytical results indicate that the Ba salt was a single compound.

On drying the Ba salt in the pistol *in vacuo* over P_2O_5 it lost weight, but quantitative water determinations were impossible. The weight loss increased steadily as the temperature in the pistol was raised from 100 to 190°, until at the latter temperature the material turned brown and was obviously decomposing.

On electrometric titration of the barium salt of 'thiodiglycol phosphate' and sodium phenyl phosphate it was found that the former bound more alkali between pH 9 and 12·2 than did the latter. The extra alkali bound was approximately one equivalent for three atoms of phosphorus.

In view of the above findings and the fact that *H* in aqueous solution readily gives rise to sulphonium salts (Herriott, 1946), the structure proposed for the barium salt of 'thiodiglycol phosphate' is



If the substance contains a sulphonium salt grouping it should be in the form of the sulphonium hydroxide when it separates from the alkaline baryta solution. The slightly high carbon content found on analysis might be due to a partial neutralization of the sulphonium hydroxide with CO_2. It seems impossible to reconcile the analytical results with any other reasonable structure, but unfortunately the proposed constitution does not readily lend itself to proof by degradation or synthesis.

For use in specific inhibition tests the Ba salt was dissolved in water and Ba removed by the careful addition of 1% Na_2SO_4 solution to slight excess. The resulting solution, after removal of $BaSO_4$ and adjustment of volume, was used as an inhibitor.

Reaction of H with serum proteins. Preparations were made from the serum proteins of the horse, rabbit, guinea pig, pig and man.

(*a*) *H-horse serum globulin* (*phosphate*, pH 8–9). Horse serum globulin solution (50 ml., containing 0·46 g. N) was mixed with 0·5M-phosphate buffer (25 ml., pH 8) and warmed to 37°. *H* (3 ml.) was added and the mixture stirred constantly and maintained at 37°. 2N-NaOH solution was added at such a rate as to keep the mixture just alkaline to phenolphthalein. After 8 hr. the reaction was finished and 19 ml. of 2N-NaOH solution had been used. The resulting solution was placed in a cellophan sac and dialysed against running tap water for 48 hr. The contents of the sac were clarified by filtering, and analyses for N and S were carried out on the solution. (At first, samples of the solution of antigen were precipitated with acetone and the precipitate, after having been washed, and dried *in vacuo*, was analysed for S and N. Since it was later found that the same values for S/N were obtained when the analyses were carried out on samples of solution, the acetone precipitation procedure was discontinued.) In each case, analyses for S and N were carried out on the original protein before treatment with *H*. Analyses for the above coupling were: original horse serum globulin solution: S/N, 0·09; coupled horse serum globulin solution: S/N, 0·34; Δ (S/N), 0·25. The final solution was stored at 5° with a few crystals of thymol as a preservative.

(*b*) *H-horse serum albumin* (*phosphate*, pH 8–9). Horse serum albumin solution (75 ml., 8·5 mg. N/ml.), 0·5M-phosphate buffer (33 ml., pH 8·0) and *H* (4 ml.) were

stirred at 37°. 2N-NaOH (27·5 ml.) was added at a rate sufficient to keep the reaction just alkaline to phenolphthalein. After 4 hr. the reaction was finished and the resulting mixture was dialysed in a cellophan sac against running tap water for 4 days. The contents of the sac were then filtered and the filtrate electrodialysed against running distilled water until no current flowed through the cell. Early in the dialysis, a gelatinous precipitate began to form and this increased in amount throughout the operation. The dialysed mixture was centrifuged and the supernatant fluid (215 ml.) had a pH of 4·5 and contained 0·07 mg. N/ml. and 0·000 mg. total P/ml.; it was discarded. The gelatinous precipitate was washed once with distilled water, centrifuged and the washing discarded. The precipitate was treated with 150 ml. water and enough N-NaOH to bring the pH to 9·0. The mixture was stored at 5° overnight when the pH had become 7·0. A trace of undissolved gel was removed by centrifuging and discarded. The supernatant solution (160 ml.) had: N, 3·43 mg./ml.; P (total), 0·25 mg./ml.; P (inorganic), 0·011 mg./ml.; S, 1·29 mg./ml.; S/N, 0·38. The original horse serum albumin solution had S/N, 0·12.

(*c*) *H-horse serum albumin* (*phosphate*, pH 7·5). A preparation of *H*-serum albumin solution was made by reacting 63 ml. of 5·3% horse serum albumin solution, 28 ml. of 0·5M-phosphate and 3·4 ml. of *H* at 37° and pH 7·5. The pH, measured by a glass electrode inserted in the reaction vessel, was kept constant at 7·5 by the addition of NaOH. After dialysis and electrodialysis, carried out as in the above preparation, the resulting solution had Δ(S/N), 0·11 and P/N, 0·070. In this preparation 5% of the phosphate was present as inorganic phosphate.

(*d*) *H-guinea pig serum* (*no buffer*, pH 8–9). Dialysed fresh guinea pig serum (22 ml.), water (20 ml.) and *H* (0·5 ml.) were stirred 8 hr. at 37°. The solution was kept alkaline to phenolphthalein by the addition of 2N-NaOH solution. The reaction mixture was dialysed against running tap water for 48 hr. and filtered; the product had Δ(S/N), 0·29.

(*e*) *Controls.* Table 1 shows the results obtained in control experiments. In each case the reactants were contained in 75 ml. water and stirred at 37° for 8 hr. at pH 8–9. The resulting solution was dialysed against running tap water for 70 hr. and then electrodialysed to zero current against distilled water.

Table 1. *Fixation of sulphur and phosphate by horse serum globulin*

Exp.	Reactants	Analyses of product S/N	P/N
LXIII	Na_2HPO_4 (0·0125 g.mol), horse serum globulin (2·8 g.)	0·12	0·005
LI	'Thiodiglycol phosphate' (0·0125 g.mol. P), horse serum globulin (2·8 g.)	0·12	0·041
LII	'Thiodiglycol phosphate' (0·0125 g.mol. P), horse serum globulin (2·8 g.) *H* (1 ml.)	0·24	0·062

The product of Exp. LII only was precipitated by the appropriate antiserum (Table 2).

Experiments with phosphatase. Two different phosphatase preparations were used; one was an aqueous extract of rat kidney, the other a solution of an active powder prepared from dog faeces (Armstrong, 1935). For use 10 mg. of this intestinal phosphatase preparation was dissolved in 20 ml. water. The rat-kidney extract was prepared by grinding fresh, decapsulated rat kidneys in a mortar with sand and five times their weight of water; the resultant brei was stored at 5° overnight and centrifuged. The supernatant liquid was diluted twenty times with water for use.

At pH 9·5 (veronal buffer of King & Delory, 1940) and 37° with 1 mg. of phosphate as 'thiodiglycol phosphate' and 5 ml. of enzyme solution in 15 ml. of digest, hydrolysis with the kidney phosphatase was 63% complete at 5 hr. and 100% complete at 22 hr.; hydrolysis with the intestinal phosphatase was 95% complete in 6 hr. and 99% complete in 22 hr.

Under the same conditions, with the *H*-horse serum albumin (phosphate) prepared at pH 8–9, as described in the previous section, the intestinal phosphatase gave 13% hydrolysis of phosphate at 22 hr. and the kidney phosphatase gave 7% hydrolysis at 22 hr. The *H*-horse serum albumin (phosphate) prepared at pH 7·5 on treatment with the intestinal phosphatase under the same conditions gave 28% hydrolysis of phosphate in 22 hr. Both these solutions, after treatment with intestinal phosphatase, were electrodialysed to zero current and analysed. The analyses showed that the S/N values had not been altered by the phosphatase treatment, but that the P/N values had been reduced by 20%.

The pH 8–9 preparation before and after phosphatase treatment was precipitated by antiserum and the precipitations were inhibited by 'thiodiglycol phosphate' (Tables 2 and 4).

Immunization experiments

Various courses of immunization were carried out by injecting antigens prepared by method (*a*) into young adult rabbits. In general, it was found that an intravenous course gave a fairly good antiserum; if this were followed by an intraperitoneal course the antiserum obtained was much weaker, but if the intraperitoneal course were followed by a second intravenous course the antiserum was once more strongly precipitating.

Antigens prepared by method (*a*) from a number of different serum proteins fell into the following order of effectiveness: pig serum globulin, pig serum, horse serum globulin, horse serum, rabbit serum, human serum globulin, human serum.

The above general conclusions were arrived at from experiments on 100 rabbits. As a result of these experiments, we settled on the following procedure which was expedient and almost invariably gave strongly precipitating antisera. Rabbits were given a course of four intravenous injections, each containing about 8 mg. of antigen nitrogen, at 4-day intervals, and were bled out 3–10 days after the last injection. The best results were obtained when coupled pig serum globulin prepared by method (*a*) was the antigen.

The immunological experiments carried out with antigens prepared by method (*c*) were too few to justify any conclusions. All attempts to produce satisfactory antisera for antigens prepared by method (*d*) failed; a large number of such attempted immunizations were made by various procedures including mixing the antigen with adjuvants according to the method of Freund & McDermott (1942).

Precipitin tests. These tests were carried out by mixing 0·5 ml. of antigen solution in increasing dilutions (1/5 to 1/2560) with 0·5 ml. of antiserum in constant dilution (1/5); the diluent was 0·85 % NaCl. All readings were made at room temperature. After the test was set up frequent readings were made to determine which tube first showed definite flocculation; this established the optimal antigen/antibody ratio. The test was then continued at room temperature overnight after which time a final reading of the precipitation was made.

Table 2 summarizes the results of precipitin tests of the sera of rabbits immunized with *H*-proteins prepared by method (*a*). These data are a representative selection from a large number of such tests.

Inhibition tests. A sample of strongly precipitating rabbit serum was produced by injecting *H*-coupled horse serum globulin prepared by method (*a*), and this antiserum was titrated with decreasing concentrations of coupled guinea pig serum prepared by method (*a*). The optimum concentration of antigen for reaction with a 1/5 dilution of antiserum was 0·025 % protein. Inhibition tests were carried out on this optimal system as follows: 0·25 ml. of solutions with decreasing concentrations of the various inhibitors were incubated for 1 hr. at 37° with 0·25 ml. of a 1/2·5 dilution of antiserum; the resulting mixtures were then tested for precipitation with 0·5 ml. of coupled guinea pig serum protein solution containing 0·025 % N. The inhibitors tested in this manner were neutral solutions of thiodiglycol, thiodiglycol and sodium phosphate, 2-hydroxy-2′-glycinodiethyl sulphide, 1:4-thiazan-4-acetic acid, 'thiodiglycol phosphate' and *H*-glycine; the di-2-(2′-amino-2′-carboxyethylthioethyl) sulphide was dissolved at pH 9 since it would not dissolve at a lower pH. The only solution which produced any inhibition was that of 'thiodiglycol phosphate'; the results of a typical inhibition test with this material are to be seen in Table 3.

Table 2. *Precipitin reactions of rabbit antisera*

(The bracketed letters (*a*), (*b*), (*d*), (*e*) refer to the subsection in which the preparation of the complex is described. h.s.a. and h.s.g. = horse serum albumin and globulin, respectively; r.s. = rabbit serum; g.p.s. = guinea pig serum; p.s.g. = pig serum globulin.)

Rabbit no.	Immunizing antigen	Antiserum (1/5) titrated with	Optimal antigen conc. (% protein)	Time of appearance of ppt.	Amount of ppt.
1321	*H*-h.s.g. (*a*)	*H*-h.s.g. (*a*) (phosphate, pH 9)	0·2	2 min.	+ + + +
1321	*H*-h.s.g. (*a*)	Normal horse serum	0·1	4 hr.	+
1321	*H*-h.g.s. (*a*)	*H*-r.s. (*a*) (phosphate, pH 9)	0·2	Immediate	+ + + +
1321	*H*-h.s.g. (*a*)	Normal rabbit serum		No ppt.	
A 24	*H*-h.s.g. (*a*)	*H*-g.p.s. (*a*) (phosphate, pH 9)	0·002	1 min.	+ + + +
A 24	*H*-h.s.g. (*a*)	*H*-g.p.s. (*d*) (no buffer, pH 9)	0·002–0·03	2 hr.	+ +
1353	*H*-r.s. (*a*)	*H*-h.s.g. (*a*) (phosphate, pH 9)	0·03	15 min.	+ + +
J 74	*H*-p.s.g. (*a*)	*H*-h.s.a. (*a*) (phosphate, pH 9)	0·2	Immediate	+ + + +
J 74	*H*-p.s.g. (*a*)	*H*-h.s.a. (*d*) (no buffer, pH 9)	No ppt. Opalescence in all tubes		
A 72	*H*-p.s.g. (*a*)	*H*-h.s.a. (*b*) (phosphate, pH 9, electrodialyzed)	0·1	1 min.	+ + + +
A 72	*H*-p.s.g. (*a*)	*H*-h.s.a. (*b*) (same as above but after phosphatase treatment)	0·03	1 min.	+ + + +
A 72	*H*-p.s.g. (*a*)	*H*-h.s.g. LII (*e*) ('thiodiglycol phosphate'; pH 9)	0·5	1 min.	+ + + +

Table 3. *Inhibition test using 'thiodiglycol phosphate'*

(Antiserum to *H*-horse serum globulin (phosphate, pH 9). Test antigen *H*-guinea pig serum (phosphate, pH 9). Each tube contained 0·25 ml. of antiserum (1/2·5), 0·25 ml. of inhibitor solution and 0·5 ml. of antigen (0·025 %). Degrees of precipitation: op., opalescence without particulation; tr., trace of precipitate; ±, +, + +, etc., increasing degrees of precipitation.)

Tube no.	1	2	3	4	5	6
Conc. of inhibitor (mg. P/ml.)	2·0	1·0	0·5	0·25	0·125	
Saline (ml.)						0·25
Precipitation observed at:						
30 min.				op.	op.	+ + +
2·5 hr.			op.	op.	op.	+ + + +
6 hr.			op.	tr.	±	+ + + +
20 hr.		tr.	+	+ +	+ +	+ + + +

Table 4. *Effect of 'thiodiglycol phosphate' on precipitin reactions*

(Bracketed letters refer to methods of preparation.)

Rabbit antiserum to	Test antigen	Inhibition
H-horse serum globulin (*a*) (phosphate, pH 9)	*H*-guinea pig serum (*a*) (phosphate, pH 9)	+
H-horse serum globulin (*a*) (phosphate, pH 9)	*H*-guinea pig serum (*d*) (no buffer, pH 9)	−
H-horse serum globulin (*a*) (phosphate, pH 9)	*H*-rabbit serum (*a*) (phosphate, pH 9)	+
H-horse serum globulin (*a*) (phosphate, pH 9)	*H*-horse serum albumin (*b*) (phosphate, pH 9, electrodialysed)	+
H-pig serum globulin (*a*)	*H*-horse serum globulin LII (*e*) ('thiodiglycol phosphate', pH 9)	+
H-pig serum globulin (*a*)	*H*-horse serum albumin (*a*) treated with phosphatase	+
Normal horse serum	Horse serum globulin	−
Hen egg albumin	Hen egg albumin	−

The effects of 'thiodiglycol phosphate' in a number of different precipitating systems are recorded in Table 4.

DISCUSSION

The qualitative effect of the presence of sodium phosphate on the reaction of *H* with protein was first suggested by the results of the inhibition tests, although a quantitative effect had been noted previously. It had been found in our experiments that four times as much *H* could be brought into reaction with a protein in the presence of sodium phosphate to yield a soluble product as when the reaction was carried out under similar conditions but with no buffer present; this was exclusive of the amount of *H* reacting with the phosphate itself. Subsequent chemical analyses then established the fact that when *H* reacted with proteins in the presence of sodium phosphate at pH 7·5 or 9, complexes were formed which contained residues of diethyl sulphide, phosphate and protein.

The presence of ester phosphate in the *H*-protein-phosphate complexes might possibly have been due to the adsorption by the protein of some 'thiodiglycol phosphate', although this seems unlikely in view of the serological findings and the vigorous dialysis treatment which some of the complexes received before analysis. In order to obtain more information on this point, however, one complex prepared at pH 8–9 and one at pH 7·5 were treated with phosphatase. Although the two phosphatase preparations both hydrolysed the 'thiodiglycol phosphate' rapidly and completely they had only a small effect on the antigens under similar conditions.

The *H*-phosphate protein complexes are highly antigenic, and the groups introduced are powerfully determinant in the immunological sense, since such complexes formed from rabbit serum are antigenic for rabbits. There is no direct evidence for the configuration of the determinant group introduced, but one possibility is that it is the same as the 'thiodiglycol phosphate' but with protein substituted for one of the phosphate groups. In this connexion ,it is important to note that some phosphate, but no sulphur, is fixed by the protein in the presence of 'thiodiglycol phosphate' and that the product does not react specifically with antiserum to *H*-protein (phosphate). This small amount of phosphate fixed in Exp. LI (Table 1) may be related to the fraction of fixed phosphate liberated by phosphatase from the complexes; in the latter case the phosphate content of the complex was reduced without a reduction in the sulphur content.

It is most unlikely that the *H*-phosphate protein complexes referred to above are the only products of reaction of *H* with protein in our experiments. A number of diethyl sulphide groups could have been introduced into the protein without the intervention of phosphate, and these could be similar to those introduced when *H* reacts with protein with no buffer present. Such a conclusion is indicated by the results obtained with antiserum A24 (Table 2). Under the conditions of our experiments, such groups showed very little capacity for producing a specific antibody response, and this lack of antigenic activity is similar to that found by Boursnell *et al.* (1946*b*) for their *H*-serum protein complexes. These findings make it very difficult to interpret the part played by *H*-protein derivatives in the phenomenon of hypersensitivity to mustard gas.

A number of experiments were carried out to explore the possibility that the reaction of mustard gas simultaneously with inorganic phosphate and protein was of some significance in the mechanism of action of mustard gas on living cells, but all the findings were negative. The results of our work probably have a bearing on some other biochemical investigations of the mechanism of mustard gas action such as the effects of mustard gas on enzymes and other proteins *in vitro*. In the case of phosphate, at least, the buffer could play a prominent part.

SUMMARY

1. Two pure substances, 1:4-thiazan-4-acetic acid and (probably) 2-hydroxy-2′-glycinodiethyl sulphide, have been isolated from the product of re-

action of mustard gas (*H*) with ethyl aminoacetate in anhydrous ethanol in the presence of sodium acetate.

2. The reaction of *H* with glycine in aqueous solution at pH 9 yields a water-soluble product with unusual properties.

3. *H* reacts extensively with sodium phosphate in aqueous solution; analyses of the reaction product indicate that it is a sulphonium salt derived from 2 mol. of *H* and containing three phosphate ester groups.

4. It has been found that when *H* reacts with serum proteins in the presence of sodium phosphate at pH 8–9, complexes are formed which contain, in addition to an increased concentration of sulphur, appreciable amounts of esterified phosphate which cannot be removed by electrodialysis.

5. When these complexes are injected intravenously into rabbits they give rise to strongly precipitating antisera.

6. The precipitin reactions between these coupled proteins and the appropriate antisera are inhibited by the reaction product of *H* and sodium phosphate, but not by a number of other derivatives of diethyl sulphide.

7. The complexes formed when *H* reacts with serum proteins at pH 8–9 with no buffer present have been found to be ineffective in producing serum precipitins in rabbits.

Microanalyses of the reaction products of mustard gas and ethyl aminoacetate were carried out by Mrs Barker through the kindness of Dr Leo Marion, Chemistry Division, National Research Council, Ottawa. Carbon and hydrogen contents of the 'thiodiglycol phosphate' were measured by Dr A. Elek, Los Angeles, California.

Acknowledgement is made to Mr J. M. Chaput for his skilful technical assistance. Thanks are due to Prof. E. G. D. Murray for his continued interest and encouragement, Prof. A. M. Wynne for helpful advice, and Dr A. R. Armstrong for a gift of intestinal phosphatase.

REFERENCES

Armstrong, A. R. (1935). *Biochem. J.* **29**, 2020.
Berenblum, I. & Wormall, A. (1939). *Biochem. J.* **33**, 75.
Banks, T. E., Boursnell, J. C., Francis, G. E., Hopwood, F. L. & Wormall, A. (1946). *Biochem. J.* **40**, 743.
Boursnell, J. C., Francis, G. E. & Wormall, A. (1946*a*). *Biochem. J.* **40**, 737.
Boursnell, J. C., Francis, G. E. & Wormall, A. (1946*b*). *Biochem. J.* **40**, 768.
Cashmore, A. E. & McCombie, H. (1923). *J. chem. Soc.* **123**, 2884.
Freund, J. & McDermott, K. (1942). *Proc. Soc. exp. Biol., N.Y.*, **49**, 548.
Hellerman, L. (1942). By communication.
Herriott, R. M. (1946). *J. gen. Physiol.* **30**, 457.
Herriott, R. M., Anson, M. L. & Northrop, J. H. (1946). *J. gen. Physiol.* **30**, 185.
King, E. J. & Delory, G. E. (1940). *Enzymologia*, **8**, 278.
Ogston, A. G. (1948). *Biochem. Soc. Symp.* no. 2, p. 2.

Investigations into Cholinesterase Levels in Serum and Cerebrospinal Fluid of Psychotic Patients

By D. F. EARLY, R. E. HEMPHILL, M. REISS and E. BRUMMEL
Biochemical and Endocrinological Research Department, Bristol Mental Hospitals

(*Received* 19 *April* 1949)

Claims have been made that the level of serum cholinesterase can be correlated with certain psychiatric conditions. This level is said to be raised in states of anxiety and depression, and lowered in catatonic stupor, schizophrenia and epileptic conditions (Jones & Tod, 1937; Jones & Stadie, 1939; Birkhäuser, 1941*a*, *b*; Richter & Lee, 1942; Butt, Comfort, Dry & Osterberg, 1942; Schütz, 1944).

The data accumulated were quite often inconsistent and did not permit definite conclusions about the role of cholinesterase in disturbances of the nervous tissue. Mendel, Hawkins & Nishikawara (1948) assumed that this might be due to the fact that at the time when these earlier investigations were reported the existence of two enzyme systems capable of hydrolysing acetylcholine was not known. Only later Mendel & Rudney (1943) and Mendel, Mundell & Rudney (1943) distinguished between the true cholinesterase and the pseudo-cholinesterase and developed methods for measuring the activities of these enzyme systems. The authors claimed that only the true cholinesterase is essential for hydrolysis of acetylcholine *in vivo*.

The investigations reported in this paper were made because of certain therapeutic possibilities envisaged, which might occur when using different substances influencing cholinesterase activity. The following points were of particular interest: (i) Whether there existed any correlation between the values of true cholinesterase and the total brain size

and the number of functioning neurons. (ii) Whether serum true cholinesterase varied in different mental disorders. (iii) What changes of true cholinesterase levels took place in the serum after electrically induced convulsions. (iv) What relationship existed between true and pseudo-cholinesterase in the serum if changes of one of them occurred. (v) Whether a relationship existed between true cholinesterase in the serum and in the cerebrospinal fluid (c.s.f.). (True cholinesterase in the c.s.f. has recently been reported (Reiss & Hemphill, 1948).)

METHODS

The cholinesterase activity was determined manometrically, using Warburg manometers, and the results expressed in μl. of CO_2 produced by 1 ml. serum or c.s.f. in 30 min. at 37·5°. The substrate concentrations used throughout the determination were: 0·3 M-acetylcholine, 0·06 M-benzoylcholine, and 0·3 M-acetyl-β-methylcholine. The substrate (0·2 ml.) was placed in the side compartment, and the c.s.f. or serum in the main compartment, with 2% bicarbonate solution (0·2 ml.) and distilled water sufficient to make the total fluid content up to 2·0 ml. For true cholinesterase determinations 1 ml. of serum was used, but for determinations with acetylcholine and benzoylcholine 0·1 or 0·05 ml. serum was sufficient. Determinations with c.s.f. required 1·6 ml.; only clear sera or c.s.f. were used. All estimations were made in duplicate, the average error being $\pm 1{\cdot}4\%$. Blood was collected from the cubital vein. The usual technique for electroshock treatment was followed, a major epileptic fit being induced with standard Ediswan apparatus. Blood samples were taken before and 2, 15 and 60 min. after the onset of the epileptic fit. The same procedure was repeated after several treatments.

RESULTS

Serum true cholinesterase determinations were made on fifty-five male chronic psychotics (aged 59–85 years). The results were divided into three groups, showing those above (*a*), inside (*b*) and below (*c*) the normal range (Table 1). Of these patients 13 (24%) showed serum true cholinesterase values within the normal range, 31 (56%) gave high values and 11 (20%) low values. Twenty-one middle-aged schizophrenics and fifteen mental defectives gave normal values. It is remarkable that the cholinesterase value of the middle-aged schizophrenics is relatively constant over a period of 3 weeks. One 9-year-old mental defective, a hydrocephalic, gave high cholinesterase values (patient no. 6 in Table 3).

Some specimen values of the fifty-five aged male psychotics for true and pseudo-cholinesterase in serum and c.f.s. are recorded in Table 2, while Table 3 shows true and pseudo-cholinesterase values of mental defectives. Both tables show that no correlation could be found between the true cholinesterase and pseudo-cholinesterase content of the serum. There is also no correlation between the cholinesterase content of serum and c.s.f.; the c.s.f. cholinesterase content could be the same whether the serum level was 170 or 5. Tables 2 and 3 show also that low levels of serum or c.s.f. cholinesterase were not related to small brains, as in defectives, or reduced cortical size, as in organic or senile dementia.

The serum cholinesterases of fifteen patients were investigated before and after electroconvulsion treatment. Representative results of five patients are recorded in Table 4. The effect of the electrically induced convulsions on the level of serum true cholinesterase was not the same for each patient. From 1 to 90 min. after shock the true cholinesterase may rise, fall or remain constant. After repeated shocks, however, the serum true cholinesterase level of the patient (taken in the morning, under standard conditions, before treatment) showed a steady decrease. This decrease is greater than spontaneous changes seen in middle-aged schizophrenics in the course of 3 weeks (cf. Table 1).

Only very slight change can be seen in the level of the pseudo-cholinesterase after electroshock treatment. The two enzyme systems react quite independently. True cholinesterase could decrease, while pseudo-cholinesterase showed a rising tendency, and vice versa.

DISCUSSION

No correlation between pseudo- and true cholinesterase was found in any of the investigations described in this paper. This is in agreement with the recent findings of Mendel *et al.* (1948), who investigated these enzyme systems in the plasma of male rats after thyroidectomy and inanition. However, considering the quite different substrates hydrolysed by these two enzyme systems no close biological correlation was to be expected.

The changes produced by electrical convulsions in the true cholinesterase level may be the expression of a regulatory mechanism starting after increased acetylcholine production has taken place. One could assume that two factors are involved in this regulatory process: (1) immediately after an electrically induced fit a great part of the enzyme system is used by the nervous system for the hydrolysis of acetylcholine and disappears from the circulation; (2) new cholinesterase is produced as a compensation, and reaches the circulation. The chronological coordination between these two processes is decisive for the kind of change—decrease or increase—in the true serum cholinesterase after electroconvulsion treatment.

Some evidence was contributed by animal experiments. We found that after intravenous injection of 4–8 mg. of acetylcholine into rabbits the true cholinesterase content of the serum 4 min. after injection decreased in four experiments, was unchanged

Table 1. *Serum true cholinesterase of some mental patients*

(Cholinesterase activities are expressed in μl. CO_2 produced by 1 ml. serum in 30 min.)

Patients and controls	No. investigated	Age		Serum true cholinesterase	
		Range	Average	Range	Mean $\pm \sigma$
Aged psychotics (*a*)*	31	59–76	66·9	62·8–233	116·2 $\pm$ 43·5
(*b*)	13	60–77	67·5	26·0–54·3	37·2 $\pm$ 8·6
(*c*)	11	65–84	75	0 –22·4	6·9 $\pm$ 8·1
Middle-aged schizophrenics	21	17–48	34·7	22·4–48·6	31·6 $\pm$ 6·47
Same patients 3 weeks later				22·4–45·4	34·7 $\pm$ 6·69
Mental defectives	15	13–54	26·3	26·4–54·8	37·5 $\pm$ 8·47
Normal controls	11	18–34	28·3	25·5–36·6	31·5 $\pm$ 4·09

* See text.

Table 2. *Serum and cerebrospinal fluid cholinesterases of some male psychotics*

(Cholinesterase activities are expressed in μl. CO_2 produced by 1 ml. serum in 30 min. ACh., acetylcholine; BCh., benzoylcholine; MCh., acetyl-β-methylcholine.)

No.	Patient	Age	Serum			Cerebrospinal fluid		
			ACh.	BCh.	MCh.	ACh.	BCh.	MCh.
4	H.M.	62	1660	690	170	10·9	4·5	5·6
6	A.E.J.	60	1671	402	167	14·7	5·4	9·0
9	F.W.B.	71	3080	1000	110	11·0	2·1	3·85
21	E.E.A.	72	3530	1457	87	15·4	0·83	5·6
26	E.N.P.	76	1822	1458	63	11·5	2·52	5·54
29	A.J.	69	2450	435	73	12·6	0	4·7
36	T.R.	73	2080	875	37	14·7	1·7	5·7
48	E.A.	78	2240	1012	8·6	15·4	8·1	8·4
50	D.M.	82	1767	662	0	21·0	0·94	9·2
52	W.G.H.	65	2685	1155	8	9·2	0	5·1
53	T.W.	71	1952	840	0	15·9	6·5	10·0

Table 3. *Serum and cerebrospinal fluid cholinesterases of mental defectives*

(Cholinesterase activities are expressed in μl. CO_2 produced by 1 ml. serum in 30 min. ACh., acetylcholine; BCh., benzoylcholine; MCh., acetyl-β-methylcholine.)

Patient no.	Age	Cerebrospinal fluid			Serum			Diagnosis
		ACh.	BCh.	MCh.	ACh.	BCh.	MCh.	
1	29	11·3	3·3	6·8	2244	830	27·6	Imbecile
2	27	17·5	3·6	6·4	2656	1075	32·2	Imbecile (epileptic)
3	24	16·5	3·95	7·85	2531	1212	49·4	Imbecile
4	24	9·15	—	5·15	2599	1155	39·6	Feeble-minded
5	28	14·3	4·2	6·8	2106	828	30·4	Imbecile
6	9	21·2	5·9	7·3	4606	2118	63·8	Hydrocephalic imbecile
7	13	9·35	2·58	—	2598	1110	36·3	Microcephalic
8	27	10·8	—	5·5	2522	835	29·5	Imbecile
9	20	9·55	4·9	4·7	2560	1080	39·7	Imbecile
10	19	8·6	2·3	3·5	2498	1058	37·9	Imbecile
11	54	11·1	0·7	6·3	2573	950	44·4	Microcephalic imbecile
12	17	9·8	3·4	4·8	2311	1388	30	Idiot
13	19	17·8	4·1	8·5	3773	1642	46·8	Imbecile
14	56	—	—	—	1961	842	26·4	Microcephalic imbecile
15	18	14·1	3·4	6·1	3123	1388	54·8	Imbecile
16	20	7·05	2·42	2·9	2873	1232	37·2	Mongol imbecile

in one, and increased in a sixth (work to be published elsewhere).

In most patients, after one electrically induced convulsion a lowered cholinesterase level could be seen on the next morning, and a further shock at this time would depress the cholinesterase level still further. We do not know to what extent this level can be depressed, nor do we know as yet whether many fits induced at short intervals would produce a rapid lowering of serum cholinesterase or, on the other hand, stimulate a compensatory mechanism, but we feel that this effect of electroshock is of

Table 4. *Serum cholinesterases of patients before and after electroconvulsion treatment*

(Cholinesterase activities are expressed in μl., CO_2 produced by 1 ml. serum in 30 min. ACh., acetylcholine; BCh., benzoylcholine; MCh., acetyl-β-methylcholine.)

					After shock								
		Before shock			After 1–3 min.			After 15 min.			After 60–90 min.		
Date	Patient	ACh.	BCh.	MCh.	ACh.	BCh.	MCh.	ACh.	BCh.	MCh.	ACh.	BCh.	MCh.
12. i. 48	H.K.K.	3518	1362	70	4100	1537	67	3694	1332	62	—	—	—
19. i. 48		3538	1443	75	3813	1774	74	3450	1312	41	—	—	—
26. i. 48		3688	1644	56	3901	1672	51	3682	1708	65	—	—	—
19. i. 48	D.R.D.	2107	939	63	2360	1097	53	2163	1093	42	—	—	—
26. i. 48		2015	908	44	2232	1065	41	2264	1081	54	—	—	—
23. ii. 48		2391	1133	34	2665	1300	20	2478	1327	32	2408	1286	47
21. vi. 48		—	1080	27	—	—	—	—	—	—	—	—	—
3. v. 48	W.	—	1186	42	—	1180	39	—	—	—	—	—	—
10. v. 48		—	1162	35	—	1195	34	—	1142	32	—	1265	34
3. v. 48	R.T.	—	1522	44	—	1583	41	—	1479	39	—	1355	42
10. v. 48		—	1242	37	—	1280	32	—	1290	35	—	1292	41
24. v. 48		—	1210	34	—	1346	36	—	1232	40	—	—	38
19. i. 48	G.K.W.	3349	1414	66	3593	1350	98	3488	1456	80	—	—	—
26. i. 48		3395	1443	57	3595	1637	70	3195	1356	55	—	—	—
2. ii. 48		2962	1188	42	3625	—	61	3355	1374	51	—	—	—

special interest in view of the recent reports from the Maudsley Hospital in London of the experimental treatment of psychotic illness and depression with anticholinesterase substances (Nevin & Rowntree, 1948).

The abnormally high serum true cholinesterase in more than half the chronic psychotic patients may also be explained on a basis of supply and demand. Comparatively slight activity of nerve cells may call for the production of correspondingly little acetylcholine, while at the same time organs responsible for the production of cholinesterase may produce a minimum amount of the enzyme, which, however, is not required. It may be postulated that this high blood cholinesterase is an expression of a disturbed balance, but it would be premature to say as yet that this is characteristic of a psychosis, but may be an effect of age, more pronounced in psychotic than in normal individuals.

The mental defectives, being both young and not psychotic, can be imagined to have a neural equilibrium more resembling normal, and, as the cholinesterase level does not seem to be related to brain size or quantity of neurons, it is reasonable to expect that the serum cholinesterase would be within the normal range, which has been found to be the case.

The complete disappearance of cholinesterase in some of the oldest patients is probably due to the cessation of the production of an enzyme which has not been required for a long period, or to a general atrophy of the centres of production.

The remarkable absence of correlation between true cholinesterase in the serum and in the cerebrospinal fluid cannot be explained at present.

SUMMARY

1. Of fifty-five aged male psychotics studied, thirty-one had a high, thirteen a normal and eleven a low serum true cholinesterase.

2. Of sixteen mental defectives investigated fifteen gave values within the normal range. A low serum or cerebrospinal fluid cholinesterase level was not related to brain size.

3. No correlation could be observed between true and pseudo-cholinesterase levels, or between serum and cerebrospinal fluid cholinesterase levels.

4. The significance of the true cholinesterase levels in the various groups is discussed.

5. From 1 to 90 min. after electroconvulsive treatment the serum cholinesterase level may be raised, lowered or unchanged, but after repeated shocks there is a decrease in this level.

6. It is suggested that the changes in true cholinesterase levels in electroconvulsion treatment cases may imply a regulatory mechanism in which (i) cholinesterase is at once used by the central nervous system to break down acetylcholine, and therefore disappears from the blood, and (ii) new cholinesterase is produced to enter the blood stream.

REFERENCES

Birkhäuser, H. (1941*a*). *Schweiz. Arch. Neurol. Psychiat.* **46**, 185.
Birkhäuser, H. (1941*b*). *Schweiz. med. Wschr.* **71**, 750.
Butt, H. R., Comfort, M. W., Dry, T. J. & Osterberg, A. E. (1942). *J. Lab. clin. Med.* **27**, 649.
Jones, M. S. & Stadie, W. C. (1939). *Quart. J. exp. Physiol.* **29**, 63.
Jones, M. S. & Tod, H. (1937). *J. ment. Sci.* **83**, 202.
Mendel, B., Hawkins, R. & Nishikawara, M. (1948). *Amer. J. Physiol.* **154**, 495.
Mendel, B., Mundell, D. B. & Rudney, H. (1943). *Biochem. J.* **37**, 473.
Mendel, B. & Rudney, H. (1943). *Biochem. J.* **37**, 59.
Nevin, S. & Rowntree, D. (1948). *J. ment. Sci.* (in the Press).
Reiss, M. & Hemphill, R. E. (1948). *Nature, Lond.*, **161**, 18.
Richter, D. & Lee, M. (1942). *J. ment. Sci.* **88**, 428, 235.
Schütz, F. (1944). *Quart. J. exp. Physiol.* **33**, 35.

Amino-acid Decarboxylases of Rat Liver

By G. H. SLOANE-STANLEY, *Department of Pharmacology, University of Oxford*

(*Received* 22 *April* 1949)

Two amino-acid decarboxylases are known to occur in the rat's liver: 3:4-dihydroxyphenylalanine (DOPA) decarboxylase (Holtz & Credner, 1942), and cysteic acid decarboxylase (Blaschko, 1942*a*). Data on the activities of these two enzymes in liver extracts from normal rats were required, for comparison with observations on animals deficient in vitamin B_6 (Blaschko, Carter, O'Brien & Sloane-Stanley, 1948). Differences were found in the cysteic acid decarboxylase activities of liver extracts from male and female rats; the present paper contains an analysis of these findings.

Page (1945) has not been able to find any DOPA decarboxylase activity in extracts from rat tissue; experiments reported below were carried out in which his findings were tested.

METHODS

The manometric measurement of decarboxylase activity followed closely the methods already described (Blaschko, 1942*a*, *b*). Extracts were prepared as follows. The tissues were ground with sand in the cold for 5–10 min., until there was no further change in the appearance of the paste; ice-cold 0·0675 M-sodium phosphate buffer of pH 7·4 (1 ml./g. of tissue) was then added, and the mixture was centrifuged for 5 min. at a relative centrifugal force of about 800 **g**, to remove the sand and a few pieces of connective tissue and undamaged cells. The opaque viscous supernatant fluid was used. The enzymic activity is expressed as q_{CO_2}, i.e. as μl. CO_2 formed/mg. fresh wt. of tissue/hr. The substrate used is shown in parentheses, thus: q_{CO_2} (DOPA) or q_{CO_2} (cysteic). In the calculation, the simplifying assumption was made that 1 ml. of extract contained the enzymic activity from 500 mg. of tissue.

For the manometric experiments, each flask contained 1·6 ml. of extract; 0·4 ml. of either water or 0·01 M-substrate was added from the side bulb immediately after the initial readings. Readings were taken for 30 min., after which time the contents of each flask were acidified by adding 0·3 ml. of 3 N-H_2SO_4 from the central well, in order to determine the amount of CO_2 retained in the solution as bicarbonate. The substrates used were either L-3:4-dihydroxyphenylalanine or L-cysteic acid. All experiments were carried out at 37·5° in an atmosphere of N_2. The enzymic activities of the extracts were calculated from the initial linear rates of formation of CO_2, corrected for retention.

Adult white rats of the Wistar strain were used throughout.

RESULTS

3:4-*Dihydroxyphenylalanine decarboxylase activity of liver extracts*

Extracts from eighteen normal rats' livers were tested; the results are given in Table 1. The mean value of q_{CO_2} (DOPA) was 0·69; there was no correlation of the enzymic activity with either sex or age of each rat. These experiments show that, under the

Table 1. 3:4-*Dihydroxyphenylalanine decarboxylase activities of rat livers*

Body wt. (g.)	Sex	q_{CO_2} (DOPA) (μl. CO_2/mg. fresh liver/hr.)
—*	—	0·438
—*	—	0·794
—	♂	0·575
155	—	0·820
165	—	0·820
170	♀	0·690
173	—	0·540
180	—	0·641
185	—	0·887
215	—	0·875
220	♀	0·820
230	—	0·706
250	—	0·643
270	♂	0·563
283	♂	0·590
305	♂	0·610
	Mean	**0·69**
	S.E. of mean	±0·03

* Each determination made with the pooled livers of two rats.

Table 2. *Cysteic acid decarboxylase activities of rat livers*

q_{CO_2} (cysteic) (μl. CO_2/mg. fresh liver/hr.)

Group no. ...	i	ii	iii	iv	v*a*	v*b*
Type of rats ...	Normal ♀	Normal ♂	Castrated ♂	Spayed ♀	Spayed ♀, untreated	Spayed ♀, +oestrone
	0·000	0·030	0·088	0·088	0·034	0·030
	0·031	0·053	0·098	0·097	0·040	0·054
	0·034	0·062	0·100	0·102	0·075	0·059
	0·044	0·088	0·100	0·135	0·088	0·060
	0·050	0·112	0·115	0·154	0·100	0·070
	0·050	0·119	0·125	0·169	0·138	0·080
	0·050	0·120	0·145	0·194	0·138	0·083
	0·050	0·125	0·152	0·231	0·145	0·094
	0·054	0·131	0·160	—	0·150	0·096
	0·056	0·137	0·169	—	0·153	0·150
	0·063	0·144	0·181	—	0·167	0·175
	0·063	0·144	0·212	—	—	—
	0·063	0·156	—	—	—	—
	0·063	0·163	—	—	—	—
	0·066	0·170	—	—	—	—
	0·073	0·172	—	—	—	—
	0·075	0·175	—	—	—	—
	0·081	0·200	—	—	—	—
	0·083	0·206	—	—	—	—
	0·090	0·206	—	—	—	—
	0·100	0·244	—	—	—	—
	0·119	0·250	—	—	—	—
	0·133	0·250	—	—	—	—
	0·240	0·260	—	—	—	—
	—	0·310	—	—	—	—
Mean	**0·070**	**0·160**	**0·137**	**0·140**	**0·112**	**0·087**
S.E. of mean (±)	0·008	0·013	0·011	0·018	0·014	0·008

conditions used, the enzymic activity of the rat's liver was easily measurable. A comparison with the data given by Blaschko (1942*b*) shows that the activity of liver extracts of other species (rhesus monkey, cat, dog, *Rana temporaria*) was much lower; the activity of guinea pig's liver extract was of the same order as that found in the present experiments with the rat.

The DOPA decarboxylase activity of two extracts of rat's kidney has also been determined; the values for q_{CO_2} (DOPA) found were 0·08 and 0·06.

Cysteic acid decarboxylase in rat's liver

Measurements of q_{CO_2} (cysteic) were made with extracts from the livers of ninety-five animals. The results are given in Table 2; the mean value for each group and the standard error of each mean are also given.

The cysteic acid decarboxylase activities of extracts from different animals varied very widely. It was found that the mean enzymic activities of extracts from the livers of animals of opposite sex were different; this was shown by the experiments of groups i and ii. The mean q_{CO_2} (cysteic) for twenty-six female rats (group i) was 0·07; this was less than half the corresponding figure for twenty-seven males (group ii), which was 0·16. The probability that this difference arose by chance is less than 1 in 1000.

In order to analyse further the sex difference in enzymic activity, experiments were carried out on castrated male rats and ovariectomized females. Twelve male rats were castrated, and the animals were killed 30 days after the operation (group iii). The mean value of q_{CO_2} (cysteic) for liver extracts from these animals was 0·137; this value was not sufficiently different from that for the normal male rats of group ii to suggest that castration had any significant effect.

The low cysteic acid decarboxylase activity of the extracts of female rats' livers was found to depend on the presence of the ovaries. Eight rats were ovariectomized and the enzymic activities of extracts of their livers were determined 25 days after the operation. These are the animals of group iv. The difference between the mean value for q_{CO_2} (cysteic) of 0·14 for this group and that for the normal male rats of group ii was not significant ($P > 0·5$); on the other hand, the difference between this value and the mean for the normal females of group i is significant ($P \ll 0·01$).

In another series of experiments twenty-two female rats were ovariectomized (group v). After a period of 3 weeks, eleven of these rats (subgroup v*b*) were treated with oestrone; the remaining eleven animals (subgroup v*a*) served as untreated controls. Each rat of subgroup v*b* received a subcutaneous injection of 2 μg. of oestrone in 0·2 ml. of olive oil/100 g. of body weight, every second or third

day. This treatment was continued for a period of about 55 days; by the time of death, each animal had received between seventeen and twenty injections. The mean value for the enzymic activity of liver extracts from the oestrone-treated ovariectomized rats was 0·118, that for the untreated ovariectomized animals was 0·227. The difference between the mean values for enzymic activity in the two subgroups was small, the probability of the difference arising by chance being 1 in 8. Yet the higher value for the untreated animals differed significantly from that for normal females ($P = 0·02$), and the difference between the mean values for the oestrone-treated animals and for normal females was not significant.

DISCUSSION

The observations on DOPA decarboxylase are in qualitative agreement with the statement of Holtz & Credner (1942) that extracts of rat's liver and kidney contain the enzyme, and they do not confirm the results of Page (1945), who failed to find the enzyme in the rat. His figures for the enzymic activity in extracts of other tissues known to contain the enzyme are lower than those previously reported (Blaschko, 1942*b*).

In the present experiments, the DOPA decarboxylase activities of rat-kidney extracts were found to be much lower than those for extracts of rat liver. This is interesting, as in all other mammalian species hitherto examined the kidney has been the organ yielding extracts of the highest activity. Quantitatively, the present data differ from those of Holtz & Credner (1942), who found a higher activity in rat-kidney extracts. This difference is probably due to the different technique employed by these authors, who used more dilute tissue extracts, made with a buffer of pH 6·5. At this pH the enzymic reaction does not run to completion; in fact, the reaction comes to a standstill so quickly that it seems doubtful whether the method is useful for the accurate measurement of enzymic activity. In the present experiments, the pH of the buffer used was 7·4, and the extracts were concentrated; the reaction was usually followed to completion and was found to be approximately quantitative.

The experiments on cysteic acid decarboxylase may explain the apparent contradiction in the results of Blaschko (1942*a*) and Medes & Floyd (1942). The latter authors were not able to demonstrate any cysteic acid decarboxylase activity in the rat's liver. The present experiments have shown that the mean enzymic activity in liver extracts from female rats is low; in some animals the activity was very low or almost absent. If Medes & Floyd (1942) used female rats for their experiments, this might account for their failure to demonstrate the presence of the enzyme.

The observations on the ovariectomized animals indicate that the low enzymic activity in liver extracts from the female rats depended on the presence of the ovaries; both series of ovariectomized animals had a high mean cysteic acid decarboxylase activity. The lowered values in the animals treated with oestrone suggest that the presence of this hormone in the normal female is connected with the low enzymic activity.

The sex difference observed was unexpected, as there is no obvious connexion between the ovaries and the reaction catalysed by cysteic acid decarboxylase. The product of the reaction is taurine. Taurine occurs in the body as a constituent of taurocholic acid, and it seems possible that the activity of the enzyme is linked up with the biosynthesis of taurocholic acid. There is good evidence that the bile salts are concerned with the emulsification and absorption of fat in the intestine (see Frazer, 1946). It is therefore possible that the rate of absorption of fat in the rat's gut is limited, through the rate of supply of taurocholic acid, by the cysteic acid decarboxylase activity of the animal's liver.

Sex differences in fat metabolism have been described by Burn & Ling (1934), who found that in rats on a diet rich in butter the injection of anterior pituitary extract caused ketosis in some of the females, but not in males. Pregnant animals fed on the butter diet developed an intense ketosis just before parturition. Furthermore, it was found by György, Rose & Shipley (1947) that ovariectomized female rats, fed on a diet rich in fat, developed fatty livers; the deposition of fat in the livers of these animals could be prevented by the administration of methionine, together with oestrone, but not by methionine alone.

Together with the present findings, these observations raise the question whether the female rat, as a result of the low cysteic acid decarboxylase activity of its liver, does normally have a low rate of fat absorption. Ovariectomy, or other factors raising the cysteic acid decarboxylase activity of the liver, might be expected to increase the rate of fat absorption to a level at which the animal would develop ketosis or a fatty liver. The effects of anterior pituitary extract, pregnancy, and ovariectomy on the rate of absorption of fat in the gut and on the cysteic acid decarboxylase activity of the liver of the female rat could be tested experimentally.

It has also been reported that the 'non-specific cholinesterase' of rat liver is distributed unevenly between the sexes; extracts from the livers of females have a higher activity, which is lowered by ovariectomy and raised again by oestrogen treatment (Sawyer & Everett, 1947). The authors consider it possible that this enzyme is concerned in fat metabolism.

SUMMARY

1. The 3:4-dihydroxyphenylalanine (DOPA) decarboxylase activities of extracts of rats' livers and kidneys, and the cysteic acid decarboxylase activities of extracts of rats' livers, have been measured.

2. The DOPA decarboxylase activity of extracts of rat liver is comparatively high, being of the same order as that previously recorded for extracts of guinea pig liver. The rat is unusual in that the DOPA decarboxylase activity of an extract of its kidney is much lower than that of its liver; the ratio is about 1/10.

3. The mean cysteic acid decarboxylase activity of extracts of the livers of male rats is about twice that of females. This difference can be abolished by the spaying of the females, but not by the castration of the males; it is partly restored by the injection of oestrone into the spayed females.

I wish to thank Prof. J. H. Burn, F.R.S., and Dr H. Blaschko for their help and encouragement, also Dr Blaschko and Dr J. M. Walker for performing operations on some of the rats. This work was done during the tenure of a Medical Research Council Studentship.

REFERENCES

Blaschko, H. (1942*a*). *Biochem. J.* **36**, 571.
Blaschko, H. (1942*b*). *J. Physiol.* **101**, 337.
Blaschko, H., Carter, C. W., O'Brien, J. R. P. & Sloane-Stanley, G. H. (1948). *J. Physiol.* **107**, 18*P*.
Burn, J. H. & Ling, H. W. (1934). *Quart. J. Pharm.* **6**, 31.
Frazer, A. C. (1946). *Physiol. Rev.* **26**, 104.
György, P., Rose, C. S. & Shipley, R. A. (1947). *Arch. Biochem.* **12**, 125.
Holtz, P. & Credner, K. (1942). *Arch. exp. Path. Pharmak.* **199**, 145.
Medes, G. & Floyd, N. (1942). *Biochem. J.* **36**, 836.
Page, E. W. (1945). *Arch. Biochem.* **8**, 145.
Sawyer, C. H. & Everett, J. W. (1947). *Amer. J. Physiol.* **148**, 675.

Methods for the Determination of *N*-Methyl-2-pyridone-5-carboxylamide and of *N*-Methyl-2-pyridone-3-carboxylamide in Human Urine

By W. I. M. HOLMAN and D. J. DE LANGE
South African Institute for Medical Research, Johannesburg

(*Received* 30 *May* 1949)

Knox & Grossman (1946, 1947) showed that *N*-methylnicotinamide can be oxidized by an enzyme in rabbit liver to *N*-methyl-2-pyridone-5-carboxylamide and isolated the latter compound from human urine after the ingestion of doses of 0·6–0·9 g. of nicotinamide per day. The conversion of part of the *N*-methylnicotinamide formed in the body into the pyridone may explain why, in the past, it has been possible to account for only a small proportion of a dose of nicotinamide in the urine in the form of then known metabolites of nicotinic acid, and why the urinary excretion of *N*-methylnicotinamide shows little correlation with the dietary intake of nicotinic acid (Perlzweig & Huff, 1945; Knox & Grossman, 1946); but before the full significance of this new metabolite of nicotinic acid can be elucidated, an accurate method for its determination in urine is required.

Holman & Wiegand (1948) synthesized *N*-methyl-2-pyridone-5-carboxylamide by methylation and oxidation of nicotinic acid, and conversion of the resulting *N*-methyl-2-pyridone-5-carboxylic acid to the corresponding amide. They found that the methylation and oxidation of nicotinamide gave *N*-methyl-2-pyridone-3-carboxylamide. The work of Knox & Grossman gave no evidence that the latter compound is excreted in human urine after administration of nicotinamide, but further information on this point is desirable.

Since pure specimens of both the isomeric amides were available, a study of their properties was made, in an effort to find reactions which could be used for their determination in urine. It was observed that the 2:3-amide could be nitrated at room temperature in the presence of concentrated sulphuric acid, and that the resulting nitro compound gave an intense and reasonably stable colour when made alkaline with sodium hydroxide. The 2:5-amide also could be nitrated, but only by the application of heat; its nitration product gave a yellow colour with weak alkalis as well as with sodium hydroxide. The colour faded rapidly in the presence of sodium hydroxide,

but was reasonably stable in the presence of weak alkalis. The compounds could be estimated colorimetrically by these reactions.

Before the methods could be applied to urine it was necessary to remove other substances which are capable either of being nitrated to derivatives which are coloured in alkaline solution (e.g. derivatives of phenol, imidazole, purine and pyrimidine), or of substances interfering in other ways, e.g. by charring in concentrated sulphuric acid. It was found possible to separate pyridones either present in, or added to, urine from almost all extraneous matter by making use of their slight solubility in chloroform and of their capacity to be adsorbed by Lloyd's reagent. By the application of these principles, procedures were worked out for the determination of both amides in human urine.

METHODS

Reagents

5 N-H_2SO_4; 0·1 N-H_2SO_4; 0·4 N-NaOH in methanol (diluted from saturated aqueous solution of NaOH with A.R. methanol); methanol (A.R.); N-NaOH; $CHCl_3$ (grade for anaesthesia); nitrating mixture (1 g. pure KNO_3 dissolved by warming in 6 ml. pure conc. H_2SO_4; freshly prepared and cooled before use); 95% (v/v) ethanol; 15% (w/v) Na_2CO_3; standard solutions of *N*-methyl-2-pyridone-3-carboxylamide and *N*-methyl-2-pyridone-5-carboxylamide (0·1 mg./ml.; the solutions were stable for at least a month when kept in a refrigerator).

Procedure

Specimens of urine were collected every 24 hr. in 25 ml. of a mixture of equal volumes of glacial acetic acid and toluene. If the total volume was <1500 ml. the specimen was diluted with water to 2 l. Specimens were usually filtered immediately after collection and the filtrate analysed without delay. Samples stored in a refrigerator for 5–10 days and then filtered gave slightly lower results, apparently owing to loss by adsorption. In such cases it was necessary to dissolve the sediment as well as possible by warming and mixing, and to omit filtration.

Determination of N-methyl-2-pyridone-5-carboxylamide

Adsorption on Lloyd's reagent. With normal urines, 25 ml. of the filtered sample were transferred to each of four 50 ml. centrifuge tubes. If the pyridone content was exceptionally high, e.g. after the ingestion of a dose of nicotinamide, it was necessary to dilute the urine to a suitable extent before analysis. Two of the tubes were labelled *U* (unknown). To each of the remaining tubes was added 1 ml. of the standard solution of *N*-methyl-2-pyridone-5-carboxylamide and the tubes were labelled *R* (recovery). From this stage onwards, until the final extraction with $CHCl_3$ had been completed, all four tubes were treated in exactly the same way. One ml. of 5 N-H_2SO_4 was added to each tube, followed by 2 g. Lloyd's reagent. The tube was stoppered, shaken for 5 min., centrifuged and the supernatant fluid discarded. To the Lloyd's reagent in the tube were added 25 ml. of 0·1 N-H_2SO_4 and, after shaking and centrifuging, the supernatant fluid was again discarded. To the Lloyd's reagent in the tube were added 10 ml. 0·4 N-NaOH in methanol and, after shaking for 5 min. and centrifuging, the supernatant fluid was transferred to a 150 ml. glass-stoppered extraction flask. The residue in the centrifuge tube was washed with 5 ml. methanol and, after mixing and centrifuging, the washings were added to the contents of the flask. The extract and washings were neutralized with glacial acetic acid (0·15 ml.) and evaporated in a boiling water bath to a volume of 1 ml., and then to dryness with the aid of suction, taking care to avoid loss by sputtering and to obtain uniform drying.

Extraction with chloroform. Exactly 0·5 ml. aqueous N-NaOH and 25 ml. $CHCl_3$ were added to the dry residue in the flask, which was stoppered (no grease) and shaken for 5 min. The mixture was filtered through a dry paper (Whatman no. 42, 12·5 cm.), taking care to minimize evaporation, into a glass-stoppered 50 ml. measuring cylinder containing 10 ml. of a dilute aqueous solution of acetic acid (2 ml. glacial acetic acid/l.) until the total volume was exactly 30 ml. The contents of the measuring cylinder were shaken for 5 min. and the layers allowed to separate.

Portions (5 ml.) of the aqueous layer were transferred from each measuring cylinder to a 7 × 1 in. Pyrex test tube and the appropriate tubes were marked *U* and *R*. The fluid remaining in the four measuring cylinders was combined, the layers were allowed to separate, and 5 ml. of the aqueous layer transferred to a fifth test tube and marked *B* (blank). The contents of the five tubes were evaporated to dryness in a boiling water bath; the residue was perfectly white in colour.

Nitration. Exactly 0·3 ml. of nitrating mixture was added to the bottom of each tube which was shaken to dissolve the residue. The tubes marked *U* and *R* were heated for 1 hr. in a boiling water bath and the tube marked *B* was left at room temperature for 1 hr.

Development of the colour. This step was carried out under carefully controlled conditions. An apparatus was used consisting of an electrically driven stirrer with a blade which fitted closely into the bottom of a 7 × 1 in. test tube, and three burettes so arranged that they could deliver water, 15% (w/v) Na_2CO_3 and ethanol, respectively, into the tube with continuous stirring. The stirrer was made by flattening the end of a glass rod and shaping to fit the tube. A freezing bath at −6° was prepared by insulating a wide-mouthed vessel of about 1 l. capacity with asbestos and filling with a solution of 100 g. NaCl in 900 ml. water. The temperature was reduced to −6° by the addition of powdered 'dry ice' and maintained by the occasional addition of a further spoonful of 'dry ice'.

After the nitration each tube was treated, in turn, as follows. The tube was clamped so that the stirrer fitted closely into its base. After switching on and ensuring that the 0·3 ml. of nitrating mixture was being mixed thoroughly and that the stirrer speed was about 300 r.p.m., the freezing bath was raised until the tube was well immersed. After stirring for 0·5 min., 1 ml. distilled water was added, drop by drop, over a period of approx. 1 min., followed by 5 ml. 15% (w/v) Na_2CO_3 drop by drop, over a period of 4–5 min. The freezing bath was lowered until the tube no longer dipped into it and 20 ml. ethanol were run in during 1 min. The stirrer was switched off, the tube removed, and the contents transferred to a centrifuge tube and centrifuged for 10 min. at 3000 r.p.m. to remove precipitated Na_2CO_3; 20 ml. of the supernatant fluid were pipetted into a flask

and diluted with 5 ml. distilled water (to prevent the appearance of turbidity on standing).

The colour intensity of each solution was measured against the blank in a photometer at 450 mμ., using a 3 cm. cell. The blank was colourless.

Calculation of results. *N*-methyl-2-pyridone-5-carboxylamide (mg. excreted/24 hr.) $= U/(R - U) \times 0{\cdot}1 \times V/v$, where U and R are the average optical densities of unknown and recovery respectively; V is total vol. (ml.) of 24 hr. sample of urine after all dilutions have been made; and v is vol. (ml.) of urine taken for analysis (25 ml.).

Determination of N-*methyl-2-pyridone-3-carboxylamide*

The procedure was very similar to that described above. Except that 1 ml. of the standard solution of the 2:3-amide was used, the technique for the adsorption on Lloyd's reagent and for the extraction with $CHCl_3$ was identical in the two methods.

After extracting with $CHCl_3$, 5 ml. of the aqueous layer were transferred from each measuring cylinder to a test tube and evaporated to dryness. A separate blank was not required. To each tube was added 0·3 ml. nitrating mixture and the residue dissolved by rotating the tube. After standing for several minutes at room temperature, the contents were diluted and neutralized at −6°, as described above. After adding 20 ml. ethanol and centrifuging off the precipitated Na_2CO_3, 20 ml. of the supernatant fluid were diluted with 5 ml. distilled water. The optical density of the solution was read against distilled water at 420 mμ., using a 3 cm. cell, before and after the addition of 0·05 ml. saturated aqueous KOH. The 24 hr. excretion in mg. was calculated by the formula given for the 2:5-amide, taking U as the average difference between the optical density of the unknown before and after the addition of KOH, and R as the corresponding difference obtained with the recovery.

RESULTS AND DISCUSSION

Accuracy of the methods

Under the conditions of nitration and colour development described above, the relationship between optical density and concentration of pyridone is linear (Fig. 1). To obtain constant colour development with the 2:5-amide, care must be taken with the dilution and neutralization, since the colour intensity is greatly influenced by the conditions of temperature and pH. Sodium carbonate gave the best results of a number of weak alkalis tested. The effectiveness of the above technique probably lies in the fact that the carbon dioxide evolved during the reaction between the carbonate and the diluted acid mixture keeps the pH at the optimum value. In the case of the 2:3-amide these conditions are not so important.

With both isomers losses occur in the adsorption on Lloyd's reagent as well as in the extraction with chloroform, but the proportion of different added amounts of either the 2:5- or the 2:3-amide which is recovered by the present technique (about 30 %) is constant for any particular sample of urine, provided that the analyses are carried out side by side in the same way, and that the amounts of added pyridone do not vary over too wide a range. There is a slight tendency for the percentage recovery to decrease as the amount of added pyridone is increased, but the effect is insignificant if the amount does not vary more than about threefold.

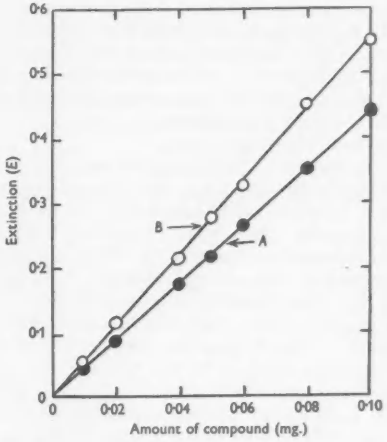


Fig. 1. Typical standard curves for *N*-methyl-2-pyridone-5-carboxylamide (*A*), and *N*-methyl-2-pyridone-3-carboxylamide (*B*). A Lumetron colorimeter was used, with 1 cm. cells.

The degree of precision of the method for the 2:5-amide, which has been examined in more detail than that for the 2:3-amide, should be adequate for most purposes. The average deviation between the optical densities of pairs of unknown solutions or recoveries has been found to be 2·5 %, and that between results for the pyridone content of urine calculated on the basis of one unknown and one recovery to be 10 %. Results calculated on average values for the optical density of two unkown solutions and two recoveries show a considerably lower degree of variability, of the order of 5 %.

The specificity of the two methods has been studied in considerable detail. Of a number of compounds which were nitrated in amounts of 0·5 mg., the following gave no colour when nitrated at room temperature or at 100°, and when made alkaline either with sodium carbonate or with potassium hydroxide: phenol, histamine, caffeine, xanthine, adenine, uric acid, guanine and thymine. Histidine, tyrosine and tyramine gave yellow colours but they were produced after nitration either at room temperature or 100°, and in the presence of either sodium carbonate or potassium hydroxide. Since

the blanks in the methods for both isomers are colourless, it is obvious that these compounds, if present in normal urine, are removed by the adsorption and extraction processes and do not interfere.

In the method for the 2:3-amide a trace of brown colour of unknown origin develops on the addition of potassium hydroxide and gives apparent values of 0·1–1·5 mg./day for the urinary excretion of this isomer. Since the colour differs widely in spectral absorption properties from the yellow colour given by the 2:3-amide, the true amounts excreted must be less than these apparent values. The fact that no significant increase in the apparent values occurred after the ingestion of a large dose of nicotinamide (Table 1), strongly suggests that the 2:3-amide is not excreted in human urine.

Although the 2:5-amide gives no colour in the method for the 2:3-amide, the latter compound, if present in urine, would interfere in the method for the 2:5-amide, since, when nitrated at 100°, it behaves in a similar manner to the 2:5-amide, giving a yellow colour with sodium carbonate. Evidence has been provided above, however, that the 2:3-amide is not present in urine. Confirmatory evidence that the method for the 2:5-amide is specific for the latter compound is afforded by the fact that the yellow colours given by a large number of samples of urine, when read against the corresponding blanks, invariably showed the same spectral absorption properties as the yellow colour derived from pure *N*-methyl-2-pyridone-5-carboxylamide.

Although both the corresponding acids, namely, *N*-methyl-2-pyridone-5-carboxylic acid and *N*-methyl-2-pyridone-3-carboxylic acid can be nitrated and give the same respective colour reactions as the amides, they are not extracted from alkaline aqueous solution by chloroform, and therefore, if present in urine, would not be included in the result. A method for the determination of the 2:5-acid in urine is being worked out and will be reported in a later communication. *N*-ethyl-2-pyridone-3-carboxylamide, *N*-benzyl-2-pyridone-3-carboxylamide, and *N*-benzyl-2-pyridone-5-carboxylic acid give similar colour reactions after nitration, but there is no reason to suspect the presence of these compounds in urine. *N*-methylnicotinamide gives no colour reaction. The specificity of the methods has been tested only with normal urines and with urines excreted by normal adults after administration of nicotinic acid and related compounds.

Application of the methods

In Table 1 are shown the results obtained by the present methods for the excretion of the 2:5- and the 2:3-amides by two healthy adult European subjects (no. 1, a male of 27 years; no. 2, a female of 36 years) on successive days before and after the oral administration of 500 mg. of nicotinamide. The corresponding values for the urinary excretion of the total acid-hydrolysable derivatives of nicotinic acid and of *N*-methylnicotinamide are also included in the table. The results show that the 2:3-amide is of no significance in nicotinic acid metabolism, but that the 2:5-amide is the most important metabolite of nicotinic acid which has been recognized up to the present time. Of the dose of 500 mg. of nicotinamide, 47–57 % was excreted as the 2:5-amide, while only 23–26 % was accounted for in the form of all other known metabolites together.

The results suggest that the sum of the daily excretions of the 2:5-amide and of *N*-methylnicotinamide may possibly afford a much better index of nicotinic acid status than any criterion which has been suggested up to the present time. Further results on the excretion of the 2:5-amide and of other metabolites of nicotinic acid after the ingestion of various derivatives of nicotinic acid will be reported in a later communication.

Table 1. *Urinary excretion of various metabolites of nicotinic acid before and after oral administration of 500 mg. of nicotinamide*

Subject no.	Metabolite of nicotinic acid	Urinary excretion (mg./day) Before dose Day 1	Before dose Day 2	After dose Day 1	After dose Day 2	After dose Day 3	Percentage of dose excreted in the urine
1	Total acid-hydrolysable derivatives*	—	2·9	19·1	2·7	2·6	3
	N-methyl-nicotinamide†	—	5·7	127	9·2	11·5	23
	N-methyl-2-pyridone-5-carboxylamide	—	7·5	256	36·6	19·9	47
	N-methyl-2-pyridone-3-carboxylamide	—	<0·9	<1·6	<0·7	<0·6	0
						Total	73
2	Total acid-hydrolysable derivatives*	0·7	0·6	13·4	0·4	0·6	3
	N-methyl-nicotinamide†	1·8	2·3	110	5·5	4·2	20
	N-methyl-2-pyridone-5-carboxylamide	6·7	6·5	295	61·3	15·6	57
	N-methyl-2-pyridone-3-carboxylamide	<0·1	<0·7	<0·9	<0·7	<0·5	0
						Total	80

* Determined by the method of Perlzweig, Levy & Sarett (1940).
† Determined by a modification of the method of Najjar & Wood (1940).

While this work was in progress an outline of a fluorimetric method for the determination of *N*-methyl-2-pyridone-5-carboxylamide in urine was reported by Rosen, Perlzweig & Handler (1948). The authors considered that their values for normal urines (5·0–13·2 mg./day) may have been somewhat low, but it is obvious that they are of the same order as those obtained by the present colorimetric method (see Table 1, and also Dean & Holman, 1949).

SUMMARY

1. Methods are described for the determination of *N*-methyl-2-pyridone-5-carboxylamide and *N*-methyl-2-pyridone-3-carboxylamide in human urine. The methods are based on the nitration of the compounds, after isolation from urine by adsorption on Lloyd's reagent followed by extraction with chloroform, and on their colorimetric determination by means of the yellow colours given by the nitro derivatives in alkaline solution.

2. The accuracy and specificity of the methods is discussed.

3. Results are shown which indicate that the 2:3-amide is of no significance in nicotinic acid metabolism, but that the 2:5-amide is an important metabolite. The 2:5-amide was excreted by two normal adults in amounts of the order of 7 mg./24 hr. After the ingestion of 500 mg. of nicotinamide, the urinary excretion of the 2:5-amide increased greatly, 47–57 % of the dose being excreted in 72 hr. as the latter compound.

4. It was possible to account for 73–80 % of an orally administered dose of 500 mg. of nicotinamide in the urine of two adult subjects as *N*-methyl-2-pyridone-5-carboxylamide, *N*-methylnicotinamide and the total acid-hydrolysable derivatives of nicotinic acid.

REFERENCES

Dean, R. F. A. & Holman, W. I. M. (1949). *Nature, Lond.*, **163**, 97.

Holman, W. I. M. & Wiegand, C. (1948). *Biochem. J.* **43**, 423.

Knox, W. E. & Grossman, W. I. (1946). *J. biol. Chem.* **166**, 391.

Knox, W. E. & Grossman, W. I. (1947). *J. biol. Chem.* **168**, 363.

Najjar, V. A. & Wood, R. W. (1940). *Proc. Soc. exp. Biol., N.Y.*, **44**, 386.

Perlzweig, W. A. & Huff, J. W. (1945). *J. biol. Chem.* **161**, 417.

Perlzweig, W. A., Levy, E. D. & Sarett, H. P. (1940). *J. biol. Chem.* **136**, 729.

Rosen, F., Perlzweig, W. A. & Handler, P. (1948). *Fed. Proc.* **7**, 181.

The Terminal Peptides of Insulin

By F. SANGER (Beit Memorial Fellow)
Biochemical Laboratory, University of Cambridge

(*Received* 1 *June* 1949)

One of the outstanding problems of protein chemistry is the elucidation of the relative positions occupied by the amino-acid residues in the protein molecule. A method for the identification of those terminal amino-acids in which the α-amino group is free has already been applied to several proteins (Sanger, 1945; Porter & Sanger, 1948). The protein is treated with 1:2:4-fluorodinitrobenzene (FDNB) and submitted to complete hydrolysis. Thereafter the *N*-2:4-dinitrophenyl (DNP) derivatives of the amino-acids are separated from each other and quantitatively estimated. By this means insulin has been shown to contain glycine and phenylalanine in the terminal position, each present to the extent of two molecules per insulin molecule of molecular weight 12,000.

The method can be adapted in determining the sequence of amino-acids which occupy positions near to the terminal amino-acid residues. For this purpose the protein is submitted to partial hydrolysis leading to the liberation of a series of *N*-2:4-dinitrophenyl (DNP) peptides. These differ from the other products of partial hydrolysis in that they are acids which can be extracted from acid solution by organic solvents and can thus be obtained relatively free from other unsubstituted peptides. The peptide mixture so produced is relatively simple and can be fractionated by chromatography on silica gel. Determination of the structure of the individual *N*-2:4-dinitrophenyl peptides then reveals the order of the amino-acid residues in proximity to the free α-amino groups. The present paper describes the results obtained by applying the method to insulin. The nature and order of the amino-acid residues contiguous to the lysyl residues have also been determined.

Since insulin contains four free α-amino groups, it was concluded that the molecule is built up of four

open polypeptide chains joined together by –S–S– bridges. These –S–S– bridges could be broken by oxidation (Sanger, 1949*a*), and on subsequent separation two main products could be obtained; an acidic fraction, *A*, which had only glycyl terminal residues, and a basic fraction, *B*, which had phenylalanyl terminal residues. For the identification of peptides described below these fractions have been used in place of the intact insulin so as to reduce the complexity of the peptide mixture dealt with.

METHODS

Materials

Insulin. The insulin used in all this work and in the earlier work (Sanger, 1945, 1949*a*) was crystalline bovine insulin having a S content of 3·2%. Fractions *A* and *B* of oxidized insulin and their DNP derivatives (referred to as DNP-*A* and DNP-*B* respectively) were prepared as previously described (Sanger, 1949*a*).

Silica gel. A number of workers who have used the silica gel solvent system procedure originally described have stated in print (Consden, Gordon, Martin & Synge, 1947; Phillips & Stephen, 1948), or in private communication, that they have experienced difficulty in that the bands have moved too rapidly down the columns. This was probably due to the properties of the silica used, although the factors enumerated below may also be contributory: (i) *Unwashed chloroform.* All $CHCl_3$ should be thoroughly washed with water to remove the ethanol added as a stabilizer. If unwashed $CHCl_3$ is used, all DNP amino-acids will travel fast on a column of silica gel. (ii) *Overloading of the column.* If too high a concentration of DNP derivative is applied to a column it will form a fast-moving band which 'tails' badly. No more than about 2 μmol. of each DNP derivative should be used on a column of diameter of 1 cm. to obtain satisfactory bands. Nevertheless, the optimum amount will depend on the particular experiment. To obtain the best separations, as are needed in the identification of a DNP amino-acid by mixed chromatograms, the smallest amount (0·1–0·2 μmol.) that can conveniently be seen on the column should be used. In quantitative estimations, a higher concentration minimizes the importance of incidental losses which are probably due to irreversible adsorption on the silica. The maximum concentration that will give a satisfactory separation should be used. In the case of the DNP peptides the limit is often set by the solubility of the derivative in the moving phase. (iii) *Water content of the silica.* The *R* values (Martin & Synge, 1941) of the derivatives vary considerably with the amount of water added to the silica, a high water content causing bands to move too rapidly. The actual capacity of the silica appears to vary from batch to batch, so that the optimal amount of water is best determined by experiments with known DNP derivatives.

All these factors apply also to the fractionation of peptides, as described below, and should be considered before discarding a gel as unsuitable for use with DNP derivatives. All gels prepared in this laboratory have been found to be satisfactory, though *R* values vary considerably from one batch to another. The gels were prepared by the method of Gordon, Martin & Synge (1943) from commercial waterglass obtained in 5 gallon drums from Joseph Crosfield Ltd., Warrington. It would seem that the source of the waterglass is more important than the actual method of preparation.

Recently Middlebrook (1949) has worked out a method for fractionating the DNP amino-acids using buffered columns (Moyle, Baldwin & Scarisbrick, 1948), which may be generally reproducible on any batch of gel. With peptides we have found that the bands tend to 'tail' rather badly on buffered columns, though they were useful for peptides that could not be dissolved easily in the solvents used on unbuffered columns, and satisfactory fractionations could often be carried out.

Fractionation of DNP peptides

The methods of fractionation of the DNP peptides are essentially the same as those used for the fractionation of DNP amino-acids (Sanger, 1945; Porter & Sanger, 1948). For a preliminary extraction of the terminal DNP peptides from other amphoteric peptides the following procedure was used. The acidic solution of the partial hydrolysate was extracted three times with equal volumes of ethyl acetate, and the combined ethyl acetate solutions extracted three times with a 1% solution of $NaHCO_3$, each bicarbonate extract being washed twice in two separating funnels with ethyl acetate. In this way one has a simple three tube counter current procedure of the type used by Craig (1944). The three bicarbonate solutions were combined, acidified and extracted again using similar counter-current procedure with ethyl acetate. This final ethyl acetate extract should contain only acidic substances. After taking to dryness the peptide mixture is fractionated on a suitable column. To describe the various solvent systems used the method and codes of Tristram (1946) are followed (Table 1). It has been found that the most generally useful solvents for the separation of the acidic terminal DNP peptides are the $CHCl_3$-*n*-butanol mixtures. The chromatographic purity of each band was usually established by running it on columns with other solvent systems.

Table 1. *Solvent systems used on silica chromatograms*

Solvent systems	Stationary phase (ml./g. silica given in brackets)	Moving phase (saturated with stationary phase)
$CHCl_3$	Water (0·5)	$CHCl_3$
CB_1	Water (0·5)	$CHCl_3$-1% (v/v) *n*-butanol
CB_5	Water (0·5)	$CHCl_3$-5% (v/v) *n*-butanol
Etc.		
Ea-HCl	N-HCl (0·5)	Ethyl acetate
Ea-pH 7	3M-phosphate buffer, pH 7 (0·6)	Ethyl acetate
M_{66}	N-HCl (0·5)	Ether-66% (v/v) methylethylketone
M_{66}-pH 6·5	3M-phosphate buffer, pH 6·5 (0·6)	Ether-66% (v/v) methylethylketone
Etc.		

Acetone-*cyclo*hexane: prepared from 1 vol. water, 3 vol. acetone and 10 vol. *cyclo*hexane (Sanger, 1945).

Sometimes it is found that a soluble compound, after being passed through a column and taken to dryness, has

become apparently insoluble. This is believed to be due to the formation of salts, and the material can generally be brought into solution once again by adding a drop of conc. HCl and a solvent in which it is readily soluble such as ether or ethyl acetate. Such a mixture is then taken to dryness and rapidly dissolved in the required solvent. This technique has also proved useful for dissolving DNP peptides (such as *A* 5) which are themselves very insoluble.

It was found that the yellow peptides containing N^5-DNP-lysine could be fractionated from one another very successfully on suitable silica columns, on which they form sharp well-defined bands. Such bands, however, are often contaminated with other colourless peptides, which can be separated by adsorption chromatography on talc from acid solution. The DNP derivatives are adsorbed firmly on the talc, whereas the simple peptides and amino-acids usually pass straight through the column.

To prepare the column, talc (5 g.) was washed several times by decantation with N-HCl. The suspension was then poured into a chromatogram tube of 1 cm. diameter and allowed to settle under slight suction. The peptide mixture was then put on the column. The DNP derivatives were adsorbed in a band at the top. After washing with about 40 ml. N-HCl, they were eluted with a mixture of 4 parts ethanol and 1 part N-HCl, in which they moved rapidly down the column. This technique could be applied to the separated DNP-lysyl peptides or to the aqueous solution of the hydrolysate before fractionation.

When it was necessary to defer the working up of a DNP peptide fraction it was usually dissolved or suspended in dilute HCl and kept in the dark. No destruction of DNP derivatives preserved in this way has been observed.

Estimation of DNP peptides

The yellow DNP amino-acids and peptides were estimated colorimetrically using the Beckman spectrophotometer. Fig. 1 shows the spectral absorption curves for DNP-phenylalanine and DNP-phenylalanyl-valine (prepared from DNP-insulin, as described below) in 1% $NaHCO_3$. It was found that the higher peptides of DNP-phenylalanine (*B* 3 and *B* 4) gave absorption curves identical with that for DNP-phenylalanyl-valine at wavelengths above 3000 A. To obtain these curves the concentration of DNP peptide was estimated from the amount of DNP-phenylalanine produced on hydrolysis. The absorption curve for DNP-glycine was almost identical with that of DNP-phenylalanine, and the curve for DNP-glycyl-glycine identical with that for DNP-phenylalanyl-valine. The DNP-glycyl peptides (*A* 4 and *A* 5) separated in the present work gave the same curve as DNP-glycyl-glycine at wavelengths >3300 A. All these DNP derivatives give identical readings at 3500 A. and the estimations have been done at this wavelength. In most experiments readings were also taken at 3900 A. and agreement between the results at the two wavelengths indicated the absence of other absorbing impurities. Table 2 shows the optical densities of 20 μM-solutions of the different DNP derivatives. Beer's law is obeyed in solutions of concentrations <50 μM. Standard curves for a number of other DNP amino-acids in 1% $NaHCO_3$ have also been determined for a wavelength of 3500 A. and have been found to differ only slightly from those of the above derivatives.

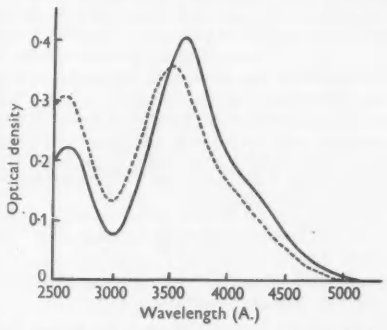


Fig. 1. Spectral absorption curves of 23·7 μM-solutions of DNP-phenylalanine (——) and DNP-phenylalanyl-valine (- - - - -) in 1% $NaHCO_3$.

Table 2. *Optical densities of 20 μM-solutions of DNP amino-acids and peptides*

DNP derivative	Solvent	Optical density ($\log I_0/I$) 3500 A.	3900 A.
DNP-glycine	1% $NaHCO_3$	0·309	0·210
DNP-phenylalanine	1% $NaHCO_3$	0·313	0·214
DNP-glycyl-glycine	1% $NaHCO_3$	0·316	0·173
DNP-phenylalanyl-valine	1% $NaHCO_3$	0·310	0·178
N^5-DNP-lysine	N-HCl	0·296	0·204
O-DNP-tyrosine	N-HCl	0·058	0·0

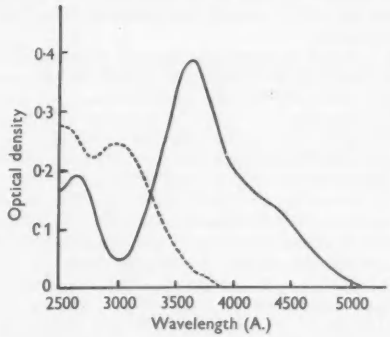


Fig. 2. Spectral absorption curves of 22 μM-solutions of N^5-DNP-lysine (——) and *O*-DNP-tyrosine (- - - - -) in N-HCl.

The determinations of the peptides containing N^5-DNP-lysine were carried out in N-HCl solutions, since N^5-DNP-lysine is unstable in alkaline solutions. The absorption curves for N^5-DNP-lysine and *O*-DNP-tyrosine are shown in Fig. 2. The curves for the lysyl peptides (*L* 2, *L* 3 and *L* 4) described below were identical with that obtained for N^5-DNP-lysine at wavelengths >3300 A. In order to avoid

interference from *O*-DNP-tyrosine, estimations of N^5-DNP-lysine and its peptides were carried out using a wavelength of 3900 A.

Identification of amino-acids present in DNP peptides

The amino-acids present in the DNP peptides were identified by paper chromatography (Consden, Gordon & Martin, 1944, 1947). After purification of the band on a suitable column, a sample containing 0·1–0·5 μmol. peptide was taken to dryness in a 50 ml. round-bottomed flask; 5 drops of 5·7 N-HCl were added and the mixture refluxed for 1 min. on a microflame to dissolve the peptide and wash down the sides of the flask. The contents were sucked into a small capillary tube using a piece of valve rubber as described by Consden, Gordon & Martin (1947). The capillary was sealed off and incubated at 105° for 24 hr. After cooling, the hydrolysate was transferred to a polythene strip and the HCl removed in a desiccator. A suitable portion of the residue was then taken for identification of the amino-acids by paper chromatography. Since only a few amino-acids were present in each peptide, it was best to use one-dimensional chromatograms on which known amino-acids were run parallel as controls. In the case of fraction *A* all the amino-acids present, except leucine and isoleucine, could be separated using the system phenol-0·3% NH_3-coal gas. Leucine and isoleucine were differentiated using 1:1 *n*-butanol-benzyl alcohol-HCN. All the amino-acids from fraction *B* could be identified using the above mixtures and *n*-butanol-acetic acid (Partridge, 1948). This latter solvent has been found to be very useful for amino-acid identification and to replace collidine for most purposes, though the R_F values vary considerably with the age of the mixture, and it is always advisable to run known amino-acids parallel to the unknowns. N^5-DNP-lysine and *O*-DNP-tyrosine can also be identified using this solvent, whereas they both run with the solvent front on phenol chromatograms.

If it is desired to identify the terminal residue as well as the other amino-acids present, the above technique can be modified in the following way. About 0·5 μmol. of the DNP peptide is hydrolysed as before, if necessary, as in the case of DNP-glycyl peptides, for a shorter period. The hydrolysate is transferred to a test tube, 2 ml. ether added and the tube shaken up. The terminal DNP amino-acid is extracted into the ether and can be identified on a suitable silica chromatogram. The aqueous solution after evaporation of the ether on a water bath is used for the identification of the other amino-acids present. In the case of peptides from fraction *B* it was often necessary to determine whether the yellow compound present in the hydrolysate was DNP-phenylalanine or N^5-DNP-lysine. This was readily accomplished by decanting the ether extract of the hydrolysate into another test tube and shaking it with a few drops of $NaHCO_3$ solution. N^5-DNP-lysine remains in the acid hydrolysate in the first test tube, whereas DNP-phenylalanine passes into the $NaHCO_3$ solution in the other test tube.

Estimation of amino-acids present in DNP peptides

In order to determine the amount of each amino-acid residue present in a peptide it was necessary to use an approximate micromethod, which could differentiate between one, two or three amino-acid residues per molecule. Quantitative paper chromatography, as used in the 'spot dilution' method of Polson, Mosley & Wyckoff (1947), was found to be very suitable and sufficiently accurate for the purpose.

After estimation of the DNP peptide in 1% $NaHCO_3$ a measured fraction (containing 0·5–1·0 μmol.) was acidified and extracted three times with ethyl acetate, each extract being washed with water to remove salts. The combined ethyl acetate solutions were taken to dryness *in vacuo* and the residue boiled under reflux with 5·7 N-HCl for a sufficient time to liberate all the terminal residue (2 hr. in the case of DNP-glycyl peptides and 4 hr. for DNP-phenylalanyl peptides). After cooling, the hydrolysate was extracted with ether, each extract being washed with water. The DNP amino-acid which was present in the ether extract was purified on a $CHCl_3$ column and estimated. The combined aqueous solution and washings were taken to dryness and hydrolysed for a further 20 hr. with 5·7 N-HCl. Excess HCl was removed by repeated evaporation *in vacuo* and the residue made to a suitable volume. Samples corresponding to various amounts between 0·05 and 0·005 μmol. peptide were then applied to a one-dimensional chromatogram and similar amounts of a standard solution of amino-acids were applied alongside the unknowns. After development of the chromatogram in a suitable solvent and treatment with ninhydrin the amount of each amino-acid could be determined by comparing the colour and size of the spots. In the case of the peptides of DNP-glycine and DNP-phenylalanine, phenol-0·3% NH_3-coal gas was a suitable solvent, and for the N^5-DNP-lysine peptides *n*-butanol-acetic acid was used.

With certain peptides it was desirable to confirm the estimations by a more accurate method and it was found that, where only a few known amino-acids were present, they could be estimated with reasonable accuracy by conversion to the DNP derivatives. The following technique was used. The aqueous hydrolysate from about 1·0 μmol. of DNP peptide, after extraction of the terminal DNP amino-acid, was taken to dryness and dissolved in 1 ml. water. $NaHCO_3$ (0·1 g.) was added and a solution of 0·1 ml. FDNB in 2 ml. ethanol. After shaking for 2 hr. the solution was taken to dryness, a few drops conc. HCl added and again taken to dryness. The residue was extracted with $CHCl_3$, CB_1 or CB_3, according to the nature of the DNP amino-acids present, and the extract put on the appropriate column. Excess FDNB moved rapidly down these columns and was discarded, while the desired DNP amino-acid bands were separated and estimated.

RESULTS

Estimation of the terminal residues of fraction A and B of oxidized insulin

As a preliminary to the following work, and also to determine the average molecular weight of the peptide chains of fractions *A* and *B* of oxidized insulin it was necessary to estimate the terminal residues of these fractions as accurately as possible.

The methods employed were those described in an earlier paper on insulin (Sanger, 1945); about 20 mg. of material were used in each experiment, which was carried out in duplicate. Since the main error is

probably due to the correction factor that has to be applied for the breakdown of the DNP amino-acids during hydrolysis, the times of hydrolysis were kept short, and small amounts of peptides present were estimated separately. In the earlier work the amount of protein present in a DNP protein was estimated from the content of amide N. This required more material than was available, so the molecular weight of the peptide was calculated from the molecular weight of the DNP derivative by assuming that one DNP group (mol.wt. 167) is attached to every free amino group and to every tyrosine and histidine residue (Porter, unpublished work). In the case of insulin this method gave the same results as the amide method.

Fraction A. For the estimation of the DNP-glycine in DNP-*A* a time of hydrolysis of 2 hr. was used and the DNP-glycine was purified on a $CHCl_3$ column. A small amount of a peptide band (probably *A* 4) was adsorbed tightly at the top of the column. This was eluted with CB_{17} and estimated separately. The results are shown in Table 3. The

Table 3. *Terminal residues of fractions A and B*

(Results corrected for breakdown during hydrolysis.)

	Fraction	μmol. DNP amino-acid/ 100 mg. DNP derivative	Mol. wt. of DNP derivative
A	DNP-glycine	27	—
	DNP-glycyl peptide	2	—
	Total	29	3450
B	DNP-phenylalanine	17	—
	DNP-phenylalanyl peptides	1·5	—
	Total	18·5	5100
	DNP-glycine	1	—
	N^5-DNP-lysine	19·5	4800

molecular weight obtained for DNP-*A* is 3450. Fraction *A* contains one free amino group and two tyrosine residues. It has no lysine or histidine (Sanger, 1949*a*), so that the DNP-*A* should contain three DNP groups, giving an additional molecular weight of 500. The molecular weight of the average chain in fraction *A* is thus 2950, which agrees well with the value of 2900 determined in the ultracentrifuge (Gutfreund & Ogston, 1949). This value corresponds to a molecular weight of 2750 for the individual chains in the intact insulin, the difference being due to the oxygen taken up during the treatment with performic acid.

Fraction B. DNP-*B* was hydrolysed for 4 hr. in boiling 5·7 N-HCl and the hydrolysate extracted with ether. The ether extract was used for the estimation of DNP-phenylalanine and the aqueous solution for N^5-DNP-lysine. The ethereal solution was taken to dryness and the residue put on a $CHCl_3$ column. Three bands were obtained. The main DNP-phenylalanine band was estimated directly. This was followed by a fainter band containing peptide *B* 2 and a small amount of DNP-glycine, which is usually present in preparations of fraction *B*. These two were separated on an acetone-*cyclo*hexane column. There was also a faint band remaining at the top of the $CHCl_3$ column, which consisted of higher DNP-phenylalanyl peptides and could be eluted with CB_{17}.

The aqueous solution, after extraction with ether, was hydrolysed for a further 20 hr. and the N^5-DNP-lysine purified on an M_{66} column and estimated.

The results for DNP-*B*, set out in Table 3, lead to an average molecular weight of 4950 on the assumption that the small amount of DNP-glycyl peptide has the same molecular weight as DNP-*A*. Fraction *B* contains two free amino groups, three tyrosine residues (estimated by difference between the tyrosine contents of insulin and fraction *A*) and two histidine residues (estimated directly on fraction *B* using histidine decarboxylase; Gale, 1945). DNP-*B* should therefore contain seven DNP groups and the molecular weight of fraction *B* would be 3800, and of the intact chains in insulin 3700. The molecular weight as determined in the ultracentrifuge was 7000 (Gutfreund & Ogston, 1949), so that it would appear that either two phenylalanyl peptide chains are joined together by some non-oxidizable cross-linkage or that in solutions of fraction *B* aggregates are formed containing two molecules. Since non-oxidizable cross-linkages are unknown, while aggregation reactions of insulin are well known (summarized by Sanger, 1949*b*), the latter explanation would seem the more probable

Isolation of DNP-phenylalanyl-valine from DNP-insulin

When DNP-insulin was hydrolysed for 2 hr. with boiling 5·7 N-HCl, a peptide band was present that moved at the same rate as DNP-glycine on a $CHCl_3$ column, but could be separated using an acetone-*cyclo*hexane column. In the experiment described by Sanger (1945), in which the DNP-amino-acids were isolated from 1·03 g. DNP-insulin, the DNP-glycine and the peptide were fractionated and the solution of the peptide in acetone-*cyclo*hexane allowed to stand overnight. It crystallized as needles as the acetone evaporated (yield 8·1 mg.).

A sample was hydrolysed and DNP-phenylalanine identified as the terminal residue. Paper chromatography showed that valine and no other amino-acid was present. By quantitative paper chromatography it was shown that there was one residue of valine per molecule of peptide and this was confirmed by estimation as the DNP derivative after purification on a $CHCl_3$ column. (Found: 3·3 % valine N as percentage of peptide; calc. for DNP-phenylalanyl-

valine, 3·2; for DNP-phenylalanyl-valyl-valine, 5·3 %.) The only possible structure for this peptide is therefore DNP-phenylalanyl-valine.

Identification of DNP peptides from fraction B

In the initial experiments, DNP-*B* was hydrolysed for 8 days with conc. HCl at 37°.

DNP-phenylalanyl peptides. The material extracted from the hydrolysate by ethyl acetate was first put through a CB_{17} column. Most of the colour travelled fast and only a few faint bands could be distinguished on the column. These were not investigated in detail. The fast-moving material was then fractionated on a CB_5 column. Three main bands were formed; *B* 3, *B* 4 and a band that moved fast; there were also a number of fainter bands moving more slowly than *B* 4 and a fairly strong one that remained at the top of the column and could be eluted with CB_{17}. Bands *B* 3 and *B* 4 were shown to be homogeneous on Ea-pH 7 columns ($R = 0{\cdot}25$ and 0·1 respectively). The material that moved fast in CB_5 consisted of DNP-phenylalanine, DNP-phenylalanyl-valine (*B* 2) and a small amount of DNP-glycine. These were separated using a $CHCl_3$ column, and the second band refractionated on acetone-*cyclo*hexane. In some experiments a band was present that was adsorbed at the top of the $CHCl_3$ column and usually split into a number of faint bands on development with CB_1. It contained a rather complex mixture of amino-acids. These bands were believed to be due to some secondary products (possibly esters), formed from the DNP peptides during the fractionation. They did not occur in most experiments, and the same phenomenon was sometimes observed with DNP-*A*.

Table 4. *Peptides containing DNP-phenylalanine*

Peptide	Column used for separation	*R* value	Amino-acids present
B 1	$CHCl_3$	0·4	None
B 2	$CHCl_3$	0·2	Valine
	Acetone-*cyclo*hexane	0·7	
B 3	CB_5	0·6	Valine, aspartic acid
B 4	CB_5	0·3	Valine, aspartic acid, glutamic acid

The amino-acids identified in the main fractions are shown in Table 4. In each case the terminal residue was DNP-phenylalanine, and quantitative paper chromatography showed that the amino-acids were present in equimolecular proportions in all the peptides. The regularly increasing complexity of these peptides suggested that they were all derived from the same peptide chain containing the terminal sequence DNP-phenylalanyl-valyl-aspartyl-glutamic acid, and this was confirmed by the following partial hydrolysis experiments.

Peptide *B* 3 (1·5 μmol.) was boiled under reflux with 5·7 N-HCl for 1 hr. and the degradation products studied chromatographically. The results are shown in Table 5. The only products of hydrolysis were DNP-phenylalanine and DNP-phenylalanyl-valine, which confirms the structure DNP-phenylalanyl-valyl-aspartic acid.

Table 5. *Partial hydrolysis of peptide* B 3

Peptide	Column used for separation	*R* value	Amino-acids present
B 3·1	$CHCl_3$	0·4	None
B 3·2	$CHCl_3$	0·2	Valine
	Acetone-*cyclo*hexane	0·7	
B 3·3	CB_5	0·6	Valine, aspartic acid

The partial hydrolysis of peptide *B* 4 was carried out for 14 days at 37° in conc. HCl. All the bands *B* 1, *B* 2, *B* 3 and unchanged *B* 4 were identified and shown to contain the same amino-acids as in Table 4. Since *B* 4 gives rise to peptide *B* 3, its structure can only be DNP-phenylalanyl-valyl-aspartyl-glutamic acid.

Peptides containing N^5*-DNP-lysine.* After extraction with ethyl acetate the aqueous solution from the hydrolysate of DNP-*B* was taken to dryness and fractionated on an M_{66} column. Three main bands were formed, and the faster one was refractionated on M_{66}-pH 6·5 to give two bands (*L* 1, *L* 2). There was also a fainter fast band that appeared to consist of higher peptides of N^5-DNP-lysine. Band *L* 2 was shown to be homogeneous on Ea-HCl ($R = 0{\cdot}2$) and CB_{30} ($R = 0{\cdot}15$) columns, and bands *L* 3 and *L* 4 were homogeneous on CB_{30} ($R = 0{\cdot}07$ and 0·04 respectively). Each peptide was purified by chromatography on talc, and the amino-acids identified and shown to be present in equimolecular amounts. The results are shown in Table 6. The terminal residues were determined by deamination with nitrosyl chloride (Consden, Gordon & Martin, 1947), and confirmed by the DNP technique. It was found that the latter method gave a rather more clear-cut result, whereas the former was much simpler to carry out. In a preliminary report of this work

Table 6. *Peptides containing* N^5*-DNP-lysine*

Peptide	*R* values: On M_{66}	*R* values: On M_{66}-pH 6·5	Amino-acids present	Terminal residue
L 1	0·6	0·3	N^5-DNP-lysine	—
L 2	0·6	0·1	N^5-DNP-lysine, alanine	N^5-DNP-lysine
L 3	0·2	—	N^5-DNP-lysine, threonine, proline	Threonine
L 4	0·13	—	N^5-DNP-lysine, threonine, proline, alanine	Threonine

(Sanger, 1948) the peptide *L*4 was described as threonyl-(N^5-DNP)-lysyl-alanine. The presence of proline had been overlooked in the paper chromatography, the yellow spot being confused with that due to N^5-DNP-lysine. It can, however, be clearly detected if the solvent *n*-butanol-acetic acid is used.

The results in Table 6 show that peptide *L*2 is N^5-DNP-lysyl-alanine. Peptides *L*3 and *L*4 were hydrolysed for 11 days with conc. HCl at 37°. The hydrolysate of *L*3 contained only the bands *L*1 and *L*3, and a faint band that moved at the same rate as *L*2 on M_{66}-pH 6·5 and contained N^5-DNP-lysine and proline. From peptide *L*4 all the bands *L*1, *L*2, *L*3 and *L*4 were obtained and were shown to contain the expected amino-acids. Since *L*4 has a terminal threonyl residue and gives rise to N^5-DNP-lysyl-alanine (*L*2) and a peptide (*L*3), containing threonine, proline and DNP-lysine, the only possible structure that can be assigned to it is threonyl-prolyl-(N^5-DNP-)-lysyl-alanine, and *L*3 must be threonyl-prolyl-(N^5-DNP-)-lysine.

Estimation of peptides containing DNP-phenylalanine and N^5-DNP-lysine in insulin

Having established the presence of the two peptide sequences, phenylalanyl-valyl-aspartyl-glutamic acid and threonyl-prolyl-lysyl-alanine, in fraction *B* of the oxidized insulin, the next problem was to estimate the yields of the various peptides from insulin, and to discover what proportion of the lysyl residues and the terminal phenylalanyl residues of insulin was present in these peptide sequences. When DNP-insulin was hydrolysed for 8 days with conc. HCl no peptides could be detected other than those which were obtained from DNP-*B* and very small amounts of DNP-glycyl peptides which did not interfere with the estimation of peptides containing DNP-phenylalanine and N^5-DNP-lysine. Almost all the terminal glycine was present as DNP-glycine itself, owing to the great lability of the glycyl peptide bond in strong acid.

About 100 mg. DNP-insulin were hydrolysed for 8 days at 37° with conc. HCl, the products fractionated as previously described for DNP-*B* and estimated colorimetrically. Peptides *B*5, *B*6 and *B*7 were obtained from bands that moved slower than *B*4 on CB_5. It was shown that each gave rise to band *B*4 on partial hydrolysis. *B*8 was composed of a number of faint bands that moved slowly on CB_{17}. These were eluted from the column with 80% (v/v) acetone, hydrolysed and the DNP-phenylalanine estimated after purification on a $CHCl_3$ column. The yields of the various peptides are shown in Table 7, expressed as moles of peptide as a percentage of the total DNP-phenylalanine in the original DNP-insulin, assuming there are exactly two phenylalanyl terminal residues per insulin molecule of molecular weight 12,000. It can be seen that 92% of the total DNP-phenylalanine is accounted for as compounds that fit into the peptide sequence DNP-phenylalanyl-valyl-aspartyl-glutamic acid, and it would seem evident that in fact all the terminal phenylalanyl residues are present in this sequence. An actual estimation of the amount of the peptide *B*4 in DNP-insulin can, however, be obtained by studying the hydrolysis of the peptide itself under

Table 7. *Yields of DNP-phenylalanyl peptides from an 8-day hydrolysate of DNP-insulin*

(Results expressed as mol. of DNP peptide as percentage of the total terminal phenylalanyl residues.)

	Peptide		Yield (%)
*B*1	DNP-phenylalanine		13
*B*2	DNP-phenylalanyl-valine	16	
*B*3	DNP-phenylalanyl-valyl-aspartic acid	13	
*B*4	DNP-phenylalanyl-valyl-aspartyl-glutamic acid	30	
	Total known peptides (*B*2, *B*3, *B*4)		59
*B*5		6	
*B*6		4	
*B*7		10	
	Total higher peptides from the same sequence		20
*B*8	Unknown mixture of peptides		6
	Total		98

Table 8. *Yields of DNP peptides from an 8-day hydrolysate of peptide* B4

Peptide	Yield from *B*4 (%)	Yield from DNP-insulin (%)
*B*1	13	13
*B*2	14	16
*B*3	12	13
*B*4	55	50–56

the conditions that were used for hydrolysing the DNP-insulin. Thus, if all the DNP-phenylalanine in DNP-insulin is combined in the single tetrapeptide sequence, then the partial hydrolysis of peptide *B*4 should give the same products in the same proportions as are obtained from DNP-insulin. This assumes that the stability of the various peptide bonds is the same whether the DNP-peptide is free or combined in the protein. While this may not be completely true it is probably a safe approximation. Table 8 shows the yields of DNP-peptides from peptide *B*4 (2 μmol.) that had been hydrolysed for 8 days in conc. HCl at 37°, compared with the yields from DNP-insulin. The figure for the amount of *B*4 obtained from DNP-insulin includes higher peptides containing this sequence. It can be seen that within the limits of the methods the two sets of figures agree. The degree of accuracy of these results probably indicates that not more than about 10% of the terminal phenylalanine of insulin is present in sequences other than that detected here. Thus, not

only do these results prove that both the phenylalanine terminal residues in the insulin molecule of molecular weight 12,000 are combined in the same tetrapeptide sequence, but they do justify the assumption of this value for the molecular weight.

Table 9. *Yields of peptides containing* N^5*-DNP-lysine from an 8-day hydrolysate of DNP-insulin*

(Results expressed as mol. of peptide as percentage of the total lysine.)

	Peptide	Yield from DNP-insulin (%)	Yield from *L* 4 (%)
L 1	N^5-DNP-lysine	14	14
L 2	N^5-DNP-lysyl-alanine	19	23
L 3	Threonyl-prolyl-(N^5-DNP)-lysine	32	32
L 4	Threonyl-prolyl-(N^5-DNP)-lysyl-alanine	23	21
	Total known peptides (*L* 2, *L* 3, *L* 4)	74	
L 5	Unknown	6	
	Total	94	90

Table 9 shows the yields of the various peptides containing N^5-DNP-lysine obtained from DNP-insulin and from a similar hydrolysate of peptide *L* 4. *L* 5 is the uninvestigated band that moved fast on an M_{66} column. The N^5-DNP-lysine in it was estimated after complete hydrolysis. The yields of the peptides from DNP-insulin make it very improbable that there is a second type of peptide containing lysine and agreement between the two sets of figures confirms this.

Identification of DNP-glycyl peptides from fraction A

Hydrolysis in concentrated hydrochloric acid. Owing to the great lability to strong acid of the peptide bond involving the carboxyl group of the terminal glycyl residues, partial hydrolysates of DNP-*A* carried out in conc. HCl contained large amounts of DNP-glycine and of long peptides that could not be investigated easily, and only poor yields of small peptides that could readily be studied. Nevertheless, it was possible to determine the structure of three of these small peptides, and as most of the work was carried out on such hydrolysates, the results are reported here, although much more satisfactory yields were obtained at a later stage when the hydrolysis was carried out in dilute acid.

DNP-*A* was hydrolysed for 24 hr. with conc. HCl at 37°, and the material that was extracted with ethyl acetate first put on a CB_{17} column. Besides the main fast-moving band there were a number of fainter bands. The strongest of these, which travelled at a gradually decreasing rate down the column, gave rise to the amino-acids leucine, serine, glutamic acid and *O*-DNP-tyrosine on hydrolysis. A peptide containing these amino-acids had been reported by Woolley (1948), and it was at first assumed that the two were the same (Sanger, 1948). On quantitative estimation, however, it was found that there were about five residues of each of the above amino-acids per residue of DNP-glycine, so that the fraction was a mixture of a small amount of a DNP-glycyl peptide (probably *A* 5), which was responsible for the colour of the band on the column, and a large amount of a non-terminal peptide containing the above four amino-acids. The exact structure of this peptide has not yet been determined, but its presence does indicate that DNP-peptides, separated by the above methods, may be contaminated by non-terminal peptides.

The coloured material that moved fast on the CB_{17} column was next put on a CB_5 column. This separated a band (*A* 4) which was run out by development with CB_8. The fast-moving material from this column was fractionated on a $CHCl_3$ column. A very strong DNP-glycine band (*A* 1) was present, and this was followed by two peptide bands (*A* 2 and *A* 3) which were developed with $CB_{0\cdot5}$ and CB_1. Band *A* 2 was relatively faint and sometimes difficult to detect. Bands *A* 3 and *A* 4 were homogeneous on Ea-pH 7 columns ($R = 0\cdot4$ and $0\cdot1$ respectively) and band *A* 4 was homogeneous on M_{66}-pH 7 ($R = 0\cdot4$) and M_{66}-pH 8 ($R = 0\cdot07$).

Table 10. *Peptides of DNP-glycine*

Peptide	Column used for separation	*R* value	Amino-acids present
A 1*	$CHCl_3$	0·2	—
A 2	$CHCl_3$	0·06	Isoleucine
	$CB_{0\cdot5}$	0·2	
	CB_1	0·6	
A 3	$CHCl_3$	0·03	Isoleucine, valine
	$CB_{0\cdot5}$	0·1	
	CB_1	0·35	
A 4	CB_5	0·25	Isoleucine, valine, glutamic acid
	CB_8	0·8	

* DNP-glycine.

Table 10 shows the amino-acid composition of the three bands. In each case the terminal residue was DNP-glycine. Estimation showed that the amino-acids in *A* 3 and *A* 4 were present in equimolecular proportions to the DNP-glycine. The composition of the peptides suggests that they are all derived from the same peptide chain containing the terminal sequence DNP-glycyl-isoleucyl-valyl-glutamic acid and that this was the structure of *A* 4 was shown by partial hydrolysis. A sample was refluxed for 12 hr. with 0·1 N-HCl. The hydrolysate contained the peptide bands *A* 2 and *A* 3 and isoleucine and valine were identified in the *A* 3.

Hydrolysis in hydrochloric acid (0·1 N). In an attempt to find a more specific method for hydro-

lysing proteins, the action of boiling 0·1 N-HCl on DNP-*A* was studied. It was found that the specificity of this hydrolytic agent was very different from that of concentrated HCl, and that the smaller DNP-peptides were produced in much better yields. It seems probable that the hydrolysis of these oxidation products in dilute acid is analogous to the hydrolyses catalysed by long-chain sulphonic acids (Steinhardt & Fugitt, 1942), the peptide chains containing $-SO_3H$ groups acting as long-chain anions and catalysing a particular type of hydrolysis which is characterized by the increased lability for the amide groups and no doubt a corresponding difference in specificity towards other peptide bonds. That the $-SO_3H$ groups were catalysing the hydrolysis is indicated by the difference in the rate of hydrolysis of insulin and oxidized insulin illustrated in Fig. 3. No such difference was found when the hydrolyses were carried out in conc. HCl in which the $-SO_3H$ groups are uncharged. In these experiments the liberation of free amino groups was followed using the Van Slyke apparatus.

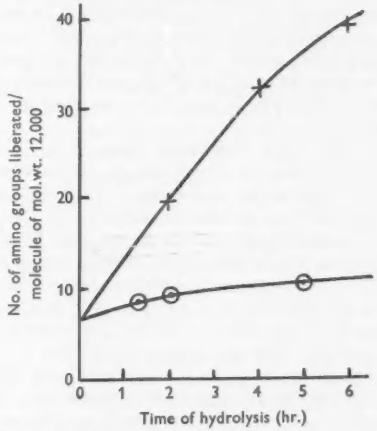


Fig. 3. Rate of hydrolysis of insulin (⊙) and oxidized insulin (+) in boiling 0·1 N-HCl.

It should be noted that the formation of diketopiperazines in dilute acid has been demonstrated (Abderhalden & Komm, 1924), so their presence was a possibility in these experiments, though the temperature employed was probably too low. The DNP derivatives could not undergo any rearrangement of this type as they contain no free amino group, and there was no evidence of any contamination of the DNP peptide bands with diketopiperazines.

In a hydrolysate of DNP-*A* that had been boiled under reflux for 12 hr. with 0·1 N-HCl there was present, besides the three previously described peptides, considerable amounts of another peptide (*A* 5) which moved with $R = 0·9$ on CB_{17} and was best purified on CB_{15} (R = approx. 0·4), though it was extremely insoluble, and only faintly coloured bands could be obtained. On an Ea-pH 6 column it gave an apparently homogeneous band with $R = 0·7$, though it 'tailed' rather badly on all buffered columns. This peptide contained DNP-glycine and the amino-acids isoleucine, valine and glutamic acid only. Quantitative paper chromatography showed that there were two residues of glutamic acid to every one residue of DNP-glycine, isoleucine and valine, and this was confirmed by estimation of the glutamic acid as the DNP derivative. No amide group was present, since no NH_3 was formed on hydrolysing for 4 hr. with boiling 2 N-HCl. Table 11 shows the results of an experiment in which *A* 5 was hydrolysed for 12 hr. with boiling 0·1 N-HCl and the hydrolysis products investigated. It can be seen that it breaks down to give peptides *A* 2, *A* 3 and *A* 4, therefore the only possible structure is DNP-glycyl-isoleucyl-valyl-glutamyl-glutamic acid.

Table 11. *Partial hydrolysis products of peptide* A 5

Peptide	Column used for separation	R value	Amino-acids present	Yield of peptide (%)
A 5·1	$CHCl_3$	0·1	—	29
A 5·2	CB_1	0·6	—	4
A 5·3	CB_1	0·35	Isoleucine, valine	10
A 5·4	CB_5	0·25	Isoleucine, valine, glutamic acid	19
A 5·5	CB_{15}	0·4	Isoleucine, valine, glutamic acid	27

Estimation of the DNP-glycyl peptides from DNP-insulin

Since DNP-insulin is insoluble in 0·1 N-HCl it was not possible to obtain a significant yield of DNP peptides by hydrolysing it directly. However, if the insulin was first oxidized and then treated with FDNB, there was very little loss of material, and the DNP-*A* present was largely soluble and gave good yields of the peptides.

In order to obtain the maximum possible yields of peptides at the expense of the yield of DNP-glycine, the hydrolysis mixture was extracted from time to time during the course of hydrolysis, so that the larger extractable peptides (*A* 4 and *A* 5) were removed and not broken down to DNP-glycine. Also unidentified fractions were hydrolysed further to give the known peptides.

As a preliminary, the breakdown of DNP-*B* under these conditions was studied. Owing to its insolubility it was not extensively hydrolysed. The main breakdown products were peptide *B* 3 and a little *B* 2. There was also a small amount of an unidentified DNP-phenylalanyl peptide that contaminated *A* 5.

Insulin (0·2 g.) was oxidized with performic acid for 30 min. (Sanger, 1949*a*). The product was precipitated with acetone and treated with FDNB for 2 hr. in the usual manner. To remove excess FDNB about 50 ml. ether and 5 ml. water were added to the reaction mixture and, after shaking and allowing to settle, the ether layer was decanted and the aqueous layer and insoluble material were extracted twice more with ether in the same way. It was then acidified with HCl and taken to dryness *in vacuo*. Excess HCl was removed by repeated evaporation *in vacuo* with water; 50 ml. 0·1 N-HCl were added and the mixture boiled under reflux for 3 hr. It was then cooled, and extracted twice with an equal volume of ethyl acetate. After removal of dissolved ethyl acetate from the aqueous layer by evaporation *in vacuo*, it was boiled for another 3 hr. and again extracted. This process was repeated six times in all, making a total time of hydrolysis of 18 hr. The ethyl acetate solutions were taken to dryness after each extraction, and the residues stored with a little water. They were all combined and the DNP peptides purified by extraction into bicarbonate as previously described. About 3 % of the DNP-glycine was lost during this treatment. The final extract containing the terminal DNP peptides was taken to dryness, the residue dissolved in 80 % ethanol and samples taken for the analyses. Several replicate analyses were performed, but not all peptides were estimated in each case.

Peptide *A* 5 was separated on a CB_{15} column. It was usually contaminated with a small amount of a DNP-phenylalanyl peptide, which was estimated from the amount of DNP-phenylalanine produced on hydrolysis of the fraction. Peptide *A* 4 was fractionated on a CB_5 column and was free from any DNP-phenylalanyl peptides. Band *B* 3 and a faint band *B* 4 moved in front of the *A* 4 band on this column, and there were traces of other unidentified DNP-phenylalanyl peptides moving more slowly. Pure bands, *A* 2 and *A* 3, were separated from a $CHCl_3$ column which was developed with CB_1. The DNP-glycine fraction obtained from the $CHCl_3$ column contained peptide *B* 2 which was separated on an acetone-*cyclo*hexane column. The yields of the various derivatives are shown in Table 12. The figures are given as percentages of the terminal glycyl residues of the original insulin taken. The corresponding yields of the DNP-phenylalanyl peptides *B* 1, *B* 2, *B* 3 and *B* 4 were 3, 9, 20 and 3 % respectively.

On the CB_{15} column much colour was present in a number of slow-moving bands which were eluted together from the column with 80 % acetone and were designated fraction *Y*. This fraction contained about 20 % of the DNP-glycine as estimated after hydrolysis. A sample was hydrolysed for 12 hr. with boiling 0·1 N-HCl, and the products estimated as for the main hydrolysate. It gave rise to the expected peptides showing that the DNP-glycine present in it was largely in the form of higher peptides of the same sequence. The yields are shown in column 4 of Table 12.

Another 8 % of the DNP-glycine was in the insoluble residue (fraction *R*) from the original dilute acid hydrolysate. This was probably present as the DNP derivative of some incompletely oxidized insulin. In order to hydrolyse it further it was first brought into solution by incubating for 1 hr. at 37° with conc. HCl. After removal of the HCl *in vacuo*, the residue was hydrolysed for a further two periods of 12 hr. with 0·1 N-HCl, and the peptides estimated as before. The yields are shown in column 5 of Table 12.

The total yields of peptides leave no doubt that both the terminal glycyl residues are combined in the same terminal peptide sequence. It was not possible to determine the amount of peptides *A* 4 or *A* 5 directly by studying their breakdown under identical conditions of hydrolysis, as was done for peptides *B* 4 and *L* 4, since the type of hydrolysis used here could not be reproduced on a peptide. The unchanged peptide would be removed on each extraction with ethyl acetate, whereas long peptides from DNP-*A* remained in the aqueous solution and were hydrolysed further. It is also probable that the long-chain charged polypeptides have a catalytic effect on the hydrolysis, which it would be difficult to reproduce exactly on a peptide. The sum of the yields of *A* 4 and *A* 5 (55 %) does, however, give direct proof that over

Table 12. *Yields of DNP-glycyl peptides from DNP-insulin*

(Results expressed as mol. of peptide as percentage of the terminal glycyl residues in the original insulin.)

Peptide	Yield from main hydrolysate	Mean	Yield from fraction *Y* (%)	Yield from fraction *R* (%)	Total yield (%)
A 1	17·5, 16	17	3·3	1·3	22
A 2	1·4	1	2·6	0·5	9
A 3	4, 5	5			
A 4	11, 11	11	2·9	1·1	15
A 5	37, 30, 38, 35	35	3·5	1·9	40
Total known peptides					64
Total					86

half of the terminal glycyl residues are in the form of the sequence glycyl-isoleucyl-valyl-glutamic acid. Table 11 shows that neither of these peptides is completely stable to this type of hydrolysis, so that the actual amount present must be considerably greater.

DISCUSSION

It has been shown that both the terminal phenylalanyl residues are combined in the insulin molecule in the form of the single tetrapeptide sequence phenylalanyl-valyl-aspartyl-glutamic acid, and that both the lysyl residues which are in the same chains are in the form of the sequence threonyl-prolyl-lysyl-alanine. Also both the terminal glycyl residues of the other two chains are present in the pentapeptide sequence glycyl-isoleucyl-valyl-glutamyl-glutamic acid. The simplest explanation of these results is that the two phenylalanyl chains are identical, and that the two glycyl chains are identical. In the phenylalanyl chains, which contain about thirty amino-acid residues, eight of the positions relative to the terminal residues and the lysyl residues are occupied by the same amino-acids in both chains. It is, of course, possible that they might differ in the nature of one or a few residues in a position in the chain not investigated in this work. As nothing is known about the principles which govern the arrangement of amino-acids along a peptide chain, such a possibility cannot be excluded. However, if one assumes that each position in the chain can be occupied by any of the sixteen different amino-acids present in these chains, the chance that two different chains would contain the same terminal tetrapeptide is about 1 in 50,000. Certain limits are obviously imposed by the specificities of the mechanisms responsible for protein synthesis. Investigations of the free amino groups of a number of proteins by the DNP technique (summarized by Sanger, 1949*c*) have shown that the terminal position in the protein chains may be occupied by a variety of different amino-acids. There appears to be no principle that defines the nature of the residue occupying this position in different proteins and it would seem probable that this would apply to other positions in the molecule. It would thus seem a reasonable conclusion from the above results that the insulin molecule is built up of two pairs of identical chains. However, if this were indeed the case, then the simplest asymmetric unit of insulin should have a molecular weight of 6000 and all the analytical figures for the amino-acids should fit such a unit. The most recent data on the amino-acid composition of insulin have been collected by Tristram (1949). While for most amino-acids the number of residues per unit of molecular weight 6000 approximates to a whole number within the limits of the methods of assay, the values for proline and isoleucine would indicate about 1·5 and 1·3 residues respectively. It is very unlikely that these estimations are more than 10% in error, so that they do cast considerable doubt on the above conclusion, and all that can be definitely stated is that the insulin molecule is built up of two pairs of very similar chains.

In contrast to the results of this work are the results obtained by Woolley (1949). Using a method similar in principle to that used here, he has reported the isolation of a number of peptides from a tryptic hydrolysate of DNP-insulin, which he believed to be DNP-glycyl peptides. He was unable, however, to demonstrate the presence of significant amounts of DNP-glycine on hydrolysis of the peptides concerned, although it could be detected in a hydrolysate of DNP-insulin. This and the results reported in the present paper would seem to indicate that the peptides isolated by Woolley were not DNP-glycyl peptides. He used ethyl acetate and butanol extracts which would be expected to contain non-terminal peptides, though it is difficult to say what the 'chromophore' group in these peptides could have been.

The methods of investigation described in this paper should be generally applicable to all proteins and peptides that contain a free amino group. Besides giving information about the structure of the proteins, the method can probably be applied as a sensitive test for the identity and homogeneity of proteins. A simple experiment in which DNP-peptide bands are separated and the amino-acids present in them identified can readily be carried out, and it is extremely unlikely that two different proteins would give the same result, probably less likely than that they would appear homogeneous by any of the known physicochemical methods of determining protein purity.

SUMMARY

1. The dinitrophenyl method for the identification of the terminal residues of proteins has been extended by the use of partial hydrolysis to the identification and estimation of terminal peptides.

2. The method was applied to insulin; and it was shown that both the terminal phenylalanyl residues are present in the form of the tetrapeptide sequence phenylalanyl-valyl-aspartyl-glutamic acid, both the lysyl residues which are in the same chains are present in the sequence threonyl-prolyl-lysyl-alanine and both the terminal glycyl residues are present in the pentapeptide sequence glycyl-isoleucyl-valyl-glutamyl-glutamic acid.

3. It is concluded that the insulin molecule is built up of two pairs of very similar, if not identical, polypeptide chains.

I wish to express my thanks to Prof. A. C. Chibnall, F.R.S., for his advice and encouragement.

REFERENCES

Abderhalden, E. & Komm, E. (1924). *Hoppe-Seyl. Z.* **134**, 121.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1947). *Biochem. J.* **41**, 590.
Consden, R., Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1947). *Biochem. J.* **41**, 596.
Craig, L. C. (1944). *J. biol. Chem.* **155**, 519.
Gale, E. F. (1945). *Biochem. J.* **39**, 46.
Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1943). *Biochem. J.* **37**, 79.
Gutfreund, H. & Ogston, A. G. (1949). *Biochem. J.* **44**, 163.
Martin, A. J. P. & Synge, R. L. M. (1941). *Biochem. J*, **35**, 1358.
Middlebrook, W. R. (1949) *Nature, Lond.*, **164**, 501.
Moyle, V., Baldwin, E. & Scarisbrick, R. (1948). *Biochem. J.* **43**, 308.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Phillips, D. M. P. & Stephen, J. M. L. (1948). *Nature, Lond.*, **162**, 152.
Polson, A., Mosley, V. M. & Wyckoff, R. W. G. (1947). *Science*, **105**, 603.
Porter, R. R. & Sanger, F. (1948). *Biochem. J.* **42**, 287.
Sanger, F. (1945). *Biochem. J.* **39**, 507.
Sanger, F. (1948). *Nature, Lond.*, **162**, 491.
Sanger, F. (1949*a*). *Biochem. J.* **44**, 126.
Sanger, F. (1949*b*). *Ann. Rep. chem. Soc.* **45**, 283.
Sanger, F. (1949*c*). *Biochem. Soc. Symp.* **3**, 21.
Steinhardt, J. & Fugitt, C. H. (1942). *Bur. Stand. J. Res., Wash.*, **29**, 315.
Tristram, G. R. (1946). *Biochem. J.* **40**, 721.
Tristram, G. R. (1949). *Advanc. protein. Chem.* **5** (in the Press).
Woolley, D. W. (1948). *Fed. Proc.* **7**, 200.
Woolley, D. W. (1949). *J. biol. Chem.* **179**, 593.

Regulation of Urinary Steroid Excretion

2. SPONTANEOUS CHANGES IN THE PATTERN OF DAILY EXCRETION IN MENTAL PATIENTS

By M. REISS, R. E. HEMPHILL, J. J. GORDON and E. R. COOK
Biochemical and Endocrinological Research Department, Bristol Mental Hospital, Fishponds, Bristol

(*Received* 17 *June* 1949)

The daily excretion of 17-ketosteroids of a large series of mental patients has been estimated for several months—in some cases for more than a year. It was observed that in some cases the rate of daily excretion was fairly constant, but in others there were wide variations, far outside the possible error of determination. Some light has been thrown on the physiological mechanism responsible by a detailed long-term study of steroid excretion in one patient with cyclical manic and depressive phases, and by the study of diurnal variations in 17-ketosteroid output in chronic schizophrenic patients before and after treatment.

EXPERIMENTAL

Estimation of steroid excretion. In all cases where the total neutral 17-ketosteroid fraction only was required, the simultaneous hydrolysis and extraction procedure of Callow, Callow, Emmens & Stroud (1939) was employed. These estimations were carried out at intervals over long periods, on 24–72 hr. urine specimens of mental patients and normal subjects. In the case of the cyclic patient, however, the more detailed procedure for fractionation and estimation of the steroids, as previously described by us (Reiss, Hemphill, Gordon & Cook, 1949), was followed.

Diurnal variations were estimated by making use of the micromethod of Drekter, Pearson, Bartczak & McGavack (1947) which requires only 10 ml. urine; such estimations were carried out in duplicate, the margin of error being rather greater than in the macromethod. It was thus possible to estimate 17-ketosteroid output on urine collections taken every 3 hr. throughout the day (7 a.m.–10 p.m.), ending with a 9 hr. night collection (10 p.m.–7 a.m.).

RESULTS

Variations in 24 *hr. excretion.* Fig. 1 illustrates the excretion rates of total 17-ketosteroids in mg./24 hr. for normal subjects, over periods of 6–14 months, and this diagram may be compared with the corresponding excretion rates for chronic schizophrenic patients, which are recorded in Fig. 2. The excretion rates for normal subjects are relatively constant, varying at the most $\pm 1{\cdot}5$ mg./24 hr. Some of the patients also show excretion rates which approach this constant pattern (e.g. nos. 7, 9, 10 and 12), but others show wide variations, which may amount to $\pm 6{\cdot}0$ mg./24 hr. These variations could not necessarily be correlated with the clinical picture. Further investigations designed to ascertain whether relationships exist between psychotic condition and constancy or inconstancy of ketosteroid excretion are at present being undertaken.

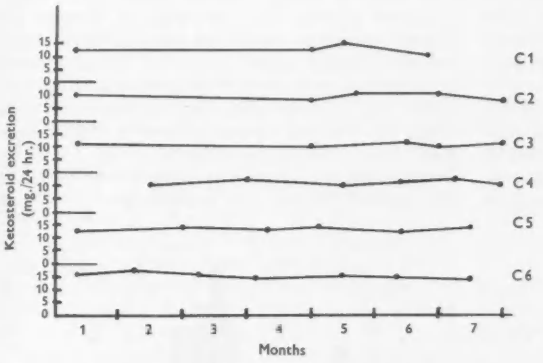


Fig. 1. Variation of urinary steroid excretion in normal subjects (72 hr. urine specimens).

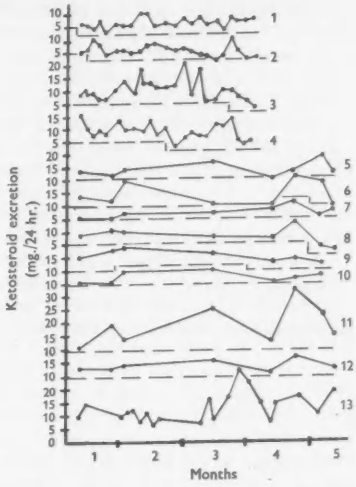


Fig. 2. Variation of urinary steroid excretion in schizophrenic subjects (48 hr. urine specimens).

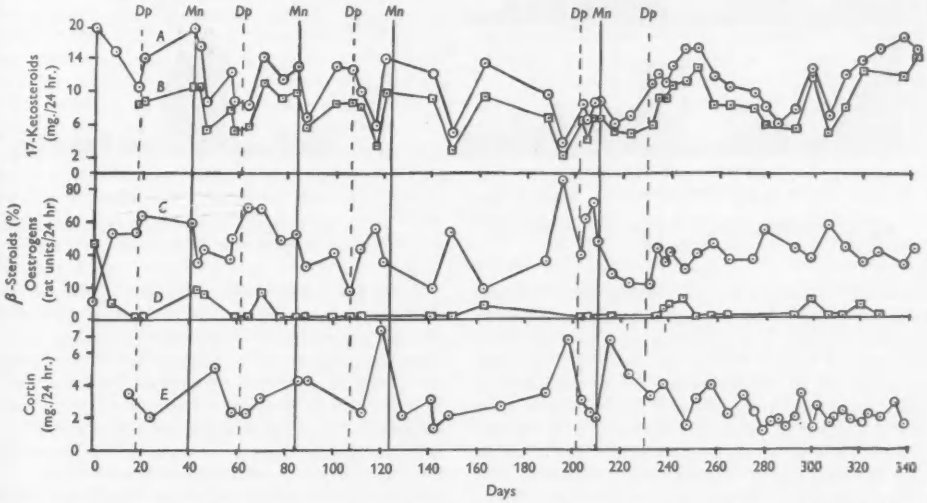


Fig. 3. Variations of urinary steroid excretion in a case of manic-depressive psychosis. *A*, total 17-ketosteroids (mg./24 hr.); *B*, ketonic fraction of 17-ketosteroids (mg./24 hr.); *C*, 3(β)-hydroxy-17-ketosteroids (% of ketonic fraction); *D*, oestrogens (rat units/24 hr.); *E*, cortin, mg./24 hr.; *Dp*, times of change into depression; *Mn*, times of change into mania.

Fig. 3 records the daily excretion rates of various steroid fractions for a 45-year-old typical manic-depressive psychotic patient, with relatively short-phase cycles. Manic phases varied between 1 and 5 weeks, and depressive phases between 6 and 24 days, the transition from one state to another being always rapid. The remission or interval of normality between manic and depressive phases was never more than a few days and merged gradually into the succeeding phase. Values are recorded for excretion of total 17-ketosteroids, ketonic fraction, percentage 3(β)-hydroxy-17-ketosteroids in the ketonic

fraction, cortin and oestrone, estimated at intervals over a period of 1 year (dates of transition from phase to phase are included). The inconstancy of the excretion rates of all these fractions is particularly marked in this case, the possible magnitude of the variations being greater than in the other cases, for corresponding periods. In as short a period as 10 days, changes of 100–300 % could occur; the cortin excretion, for instance, changed during each cycle by over 100 %, as also did that of 3(β)-hydroxy-17-ketosteroids. During the course of the year, a total 17-ketosteroid excretion varying between 2 and 18 mg./24 hr. was observed. A closer examination of the variations in different fractions shows an inverse relationship between changes in β-steroid and cortin excretion—a similar inverse relationship between β-steroid excretion and oestrone excretion was also sometimes noted. The effect was most marked in the first regular cycles, where the onset of depression appeared to be accompanied by a tendency to excretion of a high proportion of β-steroids and a low cortin excretion. Cortin excretion was usually rising at the transition into mania, when at the same time the proportion of β-steroids was falling. The clear-cut pattern of inverse relationship was followed by an irregular psychological period, during which time changes in ketosteroid excretion continued, unaccompanied by psychological changes. From September onwards, a long period of depression was maintained, in which the average β-steroid excretion was continuously high (over 20 %) and the cortin excretion comparatively low.

Diurnal variations. The variations in 17-ketosteroid excretion during the day and night were investigated in normal individuals by Pincus (1943), who reported minimal excretion during sleep and a tendency to maximal values during the hours shortly after waking, followed by a regular decline during the rest of the day. This normal pattern was shown by only a few of the psychotic patients. The results are illustrated in Fig. 4. The following deviations from the normal may be noted: (1) A greatly enhanced increase in the 'waking' output (7–10 a.m.) compared with the night output, sometimes amounting to 400 %, the corresponding figure for normal subjects being 60 % (Pincus & Hoagland, 1943). (2) Slight variations only during the day and night. (3) Maximal excretion delayed until a later time during the day, instead of in the 7–10 a.m. period. (4) A second peak in output occurring late in the day, in addition to the morning peak.

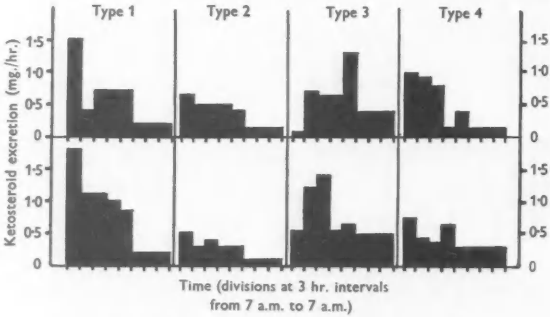


Fig. 4. Diurnal variations of urinary 17-ketosteroid excretion.

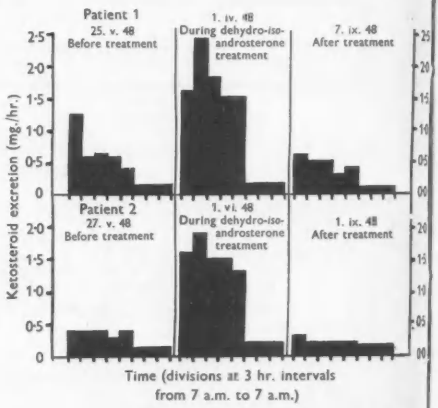


Fig. 5. Influence of dehydro*iso*androsterone on diurnal pattern of 17-ketosteroid excretion.

Types 1 and 2 are both influenced by dehydro*iso*-androsterone treatment, as illustrated in Fig. 5. Patient 2, a catatonic of 20 years' standing, underwent no change in mental state after the treatment, and it will be seen that his diurnal variation curve, which is of type 2, was not materially different after treatment had stopped, though it underwent considerable change during the course of the treatment. Patient 1, on the other hand (a hallucinated and restless schizophrenic of 16 years' standing), whose diurnal pattern is of type 1, responded well to treatment, and was subsequently discharged from hospital; at the same time, the diurnal pattern was definitely changed after cessation of treatment, approximating more to the normal.

DISCUSSION

A scrutiny of the ketosteroid excretion values of the psychotic patients, considered in relation to the known facts concerning their clinical condition,

showed that those whose excretion was constant over several months (nos. 5, 7, 8, 9, 10 and 12) were older chronic schizophrenic patients (two of them catatonic) who had been in hospital for at least 10 years. The diurnal curves of this group, as far as they have been investigated, are of type 2, showing little variation throughout the 24 hr. period. The other patients referred to in Fig. 2 were restless and hallucinated. This group showed much sharper changes in the diurnal pattern, with a degree of variation several times greater than the normal maximum of 60% observed by Pincus & Hoagland (1943) and by us. In some, the excretion peak was delayed, while in others, more than one peak could be observed. It remains to be seen whether the differentiation of diurnal curves into the types 1, 2, 3 and 4 may become useful for diagnostic purposes. It is clear, however, that the resultant of the sharply changing diurnal curves illustrated by types 1, 3 and 4 is likely to produce the greater variations in daily output.

The diurnal variation of 17-ketosteroid excretion is an expression of the adaptation by the individual to the demands of his environment and daily life. Very little adrenal cortical activity is necessary during sleep, and the most essential demand in the 24 hr. cycle is in the process of waking. It is, therefore, understandable that the excretion is at a maximum for normal subjects during the first few hours of the morning, and this adaptation mechanism is evidently disturbed in schizophrenic patients.

The daily excretion pattern of the manic-depressive patient shows that different functions of the adrenal cortex need not change in a parallel manner, since the cortin-like substances can show increases at the same time as the 3 (β)-hydroxy-17-ketosteroids are decreased, and vice versa. The antagonism between these two components was regularly observed, and changes in their values nearly always took place at times corresponding with changes in the mental state. The sudden change into depression took place while the β-steroid excretion was rising, or at a maximum, at the same time as the cortin excretion was falling; the opposite changes were seen at the commencement of the maniacal phase. For one period during the summer months of the year the typical excretion pattern was unaccompanied by the usual mental changes. Towards the autumn of the year the patient went into a prolonged depression, during which, for more than 3 months, the β-steroids remained high and the cortin excretion comparatively low.

It is important to consider the extent to which such changes in adrenal cortical activity are a primary cause, or a secondary consequence of the mental disturbance. In this connexion, the work of Allen, Broster, Vines, Patterson, Greenwood, Marrian & Butler (1939) may be taken into account. These workers claimed that paranoid psychotics with adrenogenital virilism, who showed increased ketosteroid excretion, were improved mentally after unilateral adrenalectomy, when the ketosteroid level was also lowered. These are undoubtedly special cases, and while we do not suggest that the psychiatric condition was similar to those we have studied, the physiological mechanism for the production of some of the symptoms is probably the same. Mental changes after castration are generally known, and considerable disturbances in testicular function in schizophrenic patients were demonstrated by Hemphill, Reiss & Taylor (1944). Further, in the previous paper of this series (Reiss *et al.* 1949) there was some indication that the 3 (β)-hydroxy-17-ketosteroid, dehydro*iso*androsterone, could influence the mental state. In the present study, the diurnal curve became more similar to the normal after administering dehydro*iso*androsterone in the case of the patient whose mental condition improved, but not in the case of the patient who did not improve. It may be added that, in recent months, we have accumulated results of investigations, to be described later, on depressed patients showing abnormally high β-steroid excretion, some of whom improved mentally after treatment with testosterone (which also brought about a decreased β-steroid excretion). Hoagland (1944) reported improvement in work efficiency after administration of pregn-5-en-3-ol-20-one. It is also pertinent to remember that steroid hormones may influence the brain circulation (Reiss & Golla, 1940) and may also exert a narcotic-like action (Selye, 1941).

The facts quoted above suggest that changes in hormone production may directly influence the mental state. It may be argued, however, that the response of the adrenal cortex to different demands is itself directed by vegetative brain centres, via the pituitary anterior lobe. It is perhaps permissible to visualize a cycle of events in which a disturbance of certain vegetative brain centres results in a dysfunction of the adrenal cortex—the latter in its turn influencing other brain parts and producing mental change. Further clarification of such a mechanism must await the accumulation of considerably more clinical and experimental evidence.

SUMMARY

1. Daily 17-ketosteroid excretion in the urine of mental patients, estimated at intervals for several months, showed either constant or widely varying values.

2. Different types of diurnal variation of 17-ketosteroid excretion have been recorded in mental patients.

3. A patient suffering from manic-depressive psychosis was found to excrete a high proportion of 3 (β)-hydroxy-17-ketosteroids, accompanied by a

relatively low cortin value, during most of the depressive phases. The reverse changes were usually observed during manic phases.

4. The possible relationships between steroid hormone production and mental symptoms are discussed in the light of the above findings.

The authors wish to acknowledge their indebtedness to Messrs Organon Laboratories for supplies of dehydroisoandrosterone and to Miss B. Binham and Messrs R. J. Beavan, D. J. Dell and W. G. Warren for technical assistance; also to Mr F. Knight for valuable co-operation in the collection of specimens.

REFERENCES

Allen, C., Broster, L. R., Vines, H. W. C., Patterson, J., Greenwood, A. W., Marrian, G. F. & Butler, G. C. (1939). *Brit. med. J.* i, 1220.

Callow, N. H., Callow, R. K., Emmens, C. W. & Stroud, S. W. (1939). *J. Endocrinol.* **1**, 76.

Drekter, I. J., Pearson, S., Bartczak, E. & McGavack, T. H. (1947). *J. clin. Endocrinol.* **7**, 795.

Hemphill, R. E., Reiss, M. & Taylor, A. L. (1944). *J. ment. Sci.* **90**, 681.

Hoagland, H. (1944). *J. Aviat. Med.* **15**, 98.

Pincus, G. (1943). *J. clin. Endocrinol.* **3**, 195.

Pincus, G. & Hoagland, H. (1943). *J. Aviat. Med.* **14**, 173.

Reiss, M. & Golla, Y. M. L. (1940). *J. ment. Sci.* **86**, 281.

Reiss, M., Hemphill, R. E., Gordon, J. J. & Cook, E. R. (1949). *Biochem. J.* **44**, 632.

Selye, H. (1941). *Proc. Soc. exp. Biol., N.Y.*, **46**, 116.

Optical Rotation of the Molybdate Complex of *Dextro-iso*Citric Acid

By L. V. EGGLESTON and H. A. KREBS
Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Sheffield

(*Received* 24 *June* 1949)

Owing to the high optical rotatory power of the *iso*citrate-citrate-molybdate complex, milligram quantities of *dextro-iso*citric acid can be determined polarimetrically (Martius, 1938; Jacobsohn, 1940; Martius & Leonhardt, 1943; Krebs & Eggleston, 1943).* The accuracy of the method depends, *inter alia*, on information concerning the specific rotation of this complex under the conditions of the test. Previous measurements, giving an $[\alpha]_D$ value of $-780°$, could not be regarded as highly accurate as the *iso*citric acid used for the measurements was not pure (Krebs & Eggleston, 1943). Dr H. B. Vickery has recently given us a sample of pure dimethyl *iso*citrate lactone prepared from the *iso*citric acid of *Bryophyllum* leaves (Pucher, Abrahams & Vickery, 1948). This enabled us to make more precise measurements of the rotation of the complex in the conditions used for the quantitative determination of *iso*citric acid.

EXPERIMENTAL

To prepare a stock solution of *dextro-iso*citric acid 202 mg. (1 mmol.) of the dimethyl ester lactone were dissolved in 20 ml. 0·25 N-NaOH and kept at 40° for 30 min. This treatment was found to be sufficient for the quantitative conversion of the substance into free *iso*citric acid. The stock solution was diluted as required.

The standard test conditions were as follows (see Krebs & Eggleston, 1943). To 10 ml. of the solution to be tested were added 1·25 ml. glacial acetic acid, 2·5 ml. M-sodium citrate and 11·25 ml. 29% (w/v) ammonium molybdate. (Citrate was included because it increases the rotation of the complex (Krebs & Eggleston, 1943; Martius & Leonhardt, 1943).) After mixing and filtration the solution was transferred to a 2 dm. water-jacketed Hilger polarimeter tube. Rotation readings were taken at different temperatures. The light source was a sodium lamp.

The results of the measurements at different temperatures are given in Table 1. The values for $[\alpha]_D$ are calculated for anhydrous *iso*citric acid, not for the molybdate complex (the structure of which is unknown). The concentration of *iso*citric acid in the test solution, as given in the top line of Table 1, refers to the concentration of the solution in the polarimeter tube, i.e. after the addition of the various reagents.

The data show that $[\alpha]_D$ decreases with rising temperature and also, slightly, with rising concentration of *iso*citric acid. It is therefore important in polarimetric determinations to control the temperature and to select an $[\alpha]_D$ figure for the calculation which is valid for the concentration range under test.

Further data for $[\alpha]_D^{20°}$ at ten different concentrations of *iso*citric acid are shown in Table 2. It should be noted that the absolute error of the measurements is constant (about $\pm 0·02°$ for α), and that the percentage error in α is therefore greater the smaller the value of the reading.

* The term '*dextro-iso*citric acid' is used in accordance with the conventions laid down in *Principles of Abstracting* (Bureau of Abstracts, 1948). The substance has previously been called '*d*-isocitric acid' (Pucher, Abrahams & Vickery, 1948) and '(-)*iso*citric acid' (Krebs & Eggleston, 1944).

Table 1. *Rotation of the* dextro-*isocitrate-citrate-molybdate complex*

(For test conditions see text; α_D is the rotation in degrees observed in a 2 dm. tube, $[\alpha]_D$ the specific rotation calculated for *iso*citric acid.)

Concentration of *iso*citric acid (mg./ml.)	0·48		0·96		1·92		3·84	
Temp. (°)	α_D	$[\alpha]_D$	α_D	$[\alpha]_D$	α_D	$[\alpha]_D$	α_D	$[\alpha]_D$
10	−0·80°	−834°	−1·56°	−812°	−2·98°	−776°	−5·39°	−701°
15	−0·77°	−802°	−1·50°	−781°	−2·82°	−735°	−5·18°	−675°
20	−0·75°	−781°	−1·43°	−745°	−2·66°	−693°	−4·96°	−645°
25	−0·73°	−760°	−1·38°	−719°	−2·51°	−654°	−4·75°	−618°
30	−0·70°	−729°	−1·32°	−688°	−2·35°	−612°	−4·54°	−591°
35	−0·68°	−709°	−1·26°	−657°	−2·19°	−570°	−4·33°	−564°

Table 2. *Effect of the concentration of* dextro-iso-*citric acid on* $[\alpha]_D$ *in solutions containing the* iso*citrate-citrate-molybdate complex*

(Standard conditions. 2 dm. tubes. 20°. $[\alpha]_D$ calculated for *iso*citric acid.)

*iso*Citric acid concentration (mg./ml. solution tested)	α_D observed	$[\alpha]_D$
0·240	−0·40°	−834°
0·480	−0·75°	−781°
0·576	−0·89°	−772°
0·672	−1·02°	−758°
0·768	−1·15°	−750°
0·864	−1·29°	−747°
0·960	−1·43°	−745°
1·44	−2·09°	−726°
1·92	−2·66°	−693°
3·84	−4·96°	−645°

In the previous experiments (Krebs & Eggleston, 1943) the rotation measured was about −1·80° (2 dm. tubes) at approximately 18°. According to Table 1 $[\alpha]_D^{18°}$ for these conditions is −735°, whilst it was previously assumed to be −780°. A small correction of the previously calculated *iso*-citric acid values is therefore required. The equilibrium mixture of citrate, *iso*citrate and *cis*-aconitate had been taken to contain 6·2% *iso*citrate (at 38°). The recalculation gives a value of 6·6% *iso*citrate.

SUMMARY

The optical rotatory powers of solutions containing the *iso*citrate-citrate-molybdate complex have been measured under varying conditions, in order to obtain reference values for the polarimetric determination of *dextro-iso*citric acid.

We are indebted to Dr H. B. Vickery, Connecticut Agricultural Experiment Station, New Haven, Conn., for a sample of *dextro-iso*citric acid prepared by the late Dr G. H. Pucher.

REFERENCES

Jacobsohn, K. P. (1940). *Enzymologia, Amsterdam*, **8**, 327.
Krebs, H. A. & Eggleston, L. V. (1943). *Biochem. J.* **37**, 334.
Krebs, H. A. & Eggleston, L. V. (1944). *Biochem. J.* **38**, 426.
Martius, C. (1938). *Hoppe-Seyl. Z.* **257**, 29.
Martius, C. & Leonhardt, H. (1943). *Hoppe-Seyl. Z.* **278**, 208.
Pucher, G. W., Abrahams, M. D. & Vickery, H. B. (1948). *J. biol. Chem.* **172**, 579.

The Use of Buffered Columns in the Chromatographic Separation of 2:4-Dinitrophenyl Amino-acids

By S. BLACKBURN, *Wool Industries Research Association, Torridon, Headingley, Leeds*

(*Received* 27 *June* 1949)

During an investigation of the action of papain on wool keratin, the elegant method of Sanger (Sanger, 1945; Porter & Sanger, 1948) was used to determine the end-groups of the peptides liberated by the enzyme. In this method, the terminal amino groups of proteins or polypeptides are condensed with 1:2:4-fluorodinitrobenzene and the amino-acids originally carrying free amino groups are isolated chromatographically as their coloured *N*-2:4-dinitrophenyl (DNP) derivatives after acid hydrolysis.

It was observed at an early stage that silica gels which had been successfully employed for the separation of *N*-acetyl amino-acids were of little use for the separation of DNP amino-acids. Using choloroform as the solvent, for example, practically

all the DNP amino-acids ran together as one fast band. Consden, Gordon, Martin & Synge (1947), who had a similar experience, were of the opinion that the action of Sanger's gels must be attributed to the occurrence of strong adsorption effects in addition to the effect of distribution between the phases. Sanger (1945) originally found the R values (Martin & Synge, 1941) of DNP amino-acids to vary considerably with the batch of silica, and considered that they were adsorbed on the silica to a certain extent. Phillips & Stephen (1948) have also commented on the variation between different batches of gel.

The successful use of buffered columns of silica gel in the separation of the penicillins by partition chromatography (Boon, 1948) suggested that the expedient might be useful in the separation of DNP amino-acids.

METHODS

Preparation of silica gel. The general procedure of Gordon, Martin & Synge (1943, 1944) and Tristram (1946) for the preparation of silica gel was followed. After precipitation of the gel, a considerable excess of HCl was added to the mixture, which was then allowed to stand for 3 hr. After filtration and washing, the gel was allowed to stand for 3 days in distilled water, and was again washed and dried. The gel prepared in this way was a 'non-adsorbent' gel.

Preparation of DNP amino-acids. The DNP derivatives of glycine, DL-alanine, DL-valine, DL-isoleucine, DL-leucine, L-proline, DL-phenylalanine, DL-methionine, DL-aspartic acid, DL-threonine, DL-serine and L-tryptophan were prepared by the method of Abderhalden & Blumberg (1910). Bis-DNP-L-lysine was prepared by the same method and ϵ-DNP-L-lysine according to Porter & Sanger (1948). All these derivatives were recrystallized from appropriate solvents before use and had melting points in good agreement with those in the literature. As reported by Sanger (1945), DNP-L-glutamic acid did not crystallize. DNP-DL-*glutamic acid* was prepared by the method of Abderhalden & Blumberg (1910). The cooled reaction mixture, after concentration *in vacuo* to remove the greater part of the ethanol, was diluted and extracted with ether. The heavy oil which precipitated on acidification solidified after standing overnight. It was recrystallized twice from ethyl acetate-*cyclo*hexane and had m.p. 172°. (Found: C, 42·3; H, 3·6; N, 13·6; $C_{11}H_{11}O_8N_3$ requires C, 42·2; H, 3·5; N. 13·4%.) All the above compounds were found to give single bands on appropriate silica-gel columns. The reaction between L-tyrosine and 1:2:4-chlorodinitrobenzene gave a substance forming a single band on a buffered $CHCl_3$ column. This was presumably bis-DNP-L-tyrosine, since the compound formed by the action of 1:2:4-fluorodinitrobenzene on L-tyrosine had a similar rate (Sanger, 1945).

Preparation of columns. In general the customary technique of partition chromatography was employed, 66% by weight of a suitable buffer solution being added to the dry gel before pouring the column. The buffers employed are conveniently distinguished as A, B, F and G. Buffer A was a 2M-solution of NaH_2PO_4. Buffers F, B and G were Sørensen's phosphate buffers (Britton, 1942, Table 76) of pH 6·47, 6·98 and 6·24 respectively. The concentrations of salts in these buffers were increased so that the molar concentrations of H_3PO_4 in them were respectively 0·25, 0·5 and 0·25. The pH of buffers A, B, F and G, as measured by the glass electrode, were 3·72, 6·61, 6·16 and 5·95 respectively.

It was observed during the course of this work that DNP amino-acids tended to decompose if exposed to sunlight on the column. Solutions of DNP amino-acids in solvents such as $CHCl_3$ also tended to decompose when exposed for long periods to light, particularly sunlight. The solutions then gave rise to additional bands on the chromatogram. The practice was therefore adopted of evaporating the solvent as soon as the band under consideration had run out of the column, and where necessary refractionation was carried out without undue delay. Ethanol solutions of DNP amino-acids left for some weeks in a cupboard were found to give rise to fast yellow bands on columns in addition to the bands due to the DNP amino-acid.

RESULTS

Using buffered columns of silica gel and water-saturated chloroform as solvent, mixtures of DNP amino-acids can readily be separated into distinct bands. By varying the pH of the buffer, and hence the pH of the aqueous phase, the band rate of any particular DNP amino-acid can be varied within wide limits. As the pH of the aqueous phase of the column is increased, the band rate of any particular DNP amino-acid decreases. The band rates can easily be made slow enough to separate DNP amino-acids by increasing the pH of the buffer.

Table 1 gives the band rates of a number of DNP amino-acids on columns buffered to different pH values in chloroform and n-butanol-chloroform mixtures. The behaviour of DNP-hydroxyproline was not studied, since it was known to be absent from the mixtures it was desired to investigate. Over the pH range studied, the variation of band rate with pH of the column is most marked in the case of the slower moving DNP amino-acids. Using a single solvent such as chloroform in conjunction with different buffered columns, it is possible to effect separation of DNP amino-acids which cannot be accomplished by the use of a single unbuffered column with the same solvent. The use of buffered columns largely eliminates the variations in behaviour between batches of silica gel which previous workers have commented on. A number of solvent systems have been found useful when employed with buffered columns. These systems and the type of separation they will accomplish are briefly described below. A description is also given of the method of separation of DNP amino-acid mixtures based on these solvents and buffers.

Chloroform and butanol-chloroform columns

Buffer A-$CHCl_3$ columns are useful for the separation of DNP-monoaminomonocarboxylic acids from DNP-glutamic acid, DNP-aspartic acid, DNP-threonine and DNP-serine. These columns will also separate DNP-glutamic acid from

Table 1. *Band rates (R values) of DNP amino-acids in chloroform and butanol-chloroform*

DNP amino-acid	*R* values: $CHCl_3$, buffer *A*	$CHCl_3$, buffer *B*	3% butanol-$CHCl_3$, buffer *G*	17% butanol-$CHCl_3$, buffer *B*
DNP-leucine	Fast	0·7	—	—
DNP-valine	Fast	0·5	—	—
DNP-phenylalanine	Fast	0·7	—	—
DNP-methionine	Fast	0·5	—	—
DNP-proline	Fast	0·3	—	—
DNP-alanine	Fast	0·2	—	0·5
DNP-tryptophan	0·6	0·4	Fast	—
DNP-glycine	0·9	0·03	0·5	0·2
Bis-DNP-lysine	0·7	0·06	Fast	Fast
Bis-DNP-tyrosine	Fast	0·07	0·9	Fast
DNP-threonine	0·3	Slow	0·18	0·15
DNP-serine	0·11	Slow	0·08	0·07
DNP-glutamic acid	0·17	Slow	0·2	Slow
DNP-aspartic acid	0·06	Slow	Slow	Slow

DNP-aspartic acid and DNP-threonine from DNP-serine. Buffer *B*-$CHCl_3$ columns will separate DNP-alanine from DNP-glycine and DNP-valine from DNP-alanine. The band rates of DNP amino-acids in $CHCl_3$ on columns buffered to a more alkaline pH (Sørensen's pH 8 phosphate buffer, 0·25M (Britton, 1942, Table 76) pH measured by glass electrode 7·84) were similar to those on buffer *B*-$CHCl_3$ columns. Use of these columns did not separate those DNP amino-acids not separated on buffer *B*-$CHCl_3$ columns. The rates of DNP amino-acids on buffer *B*-$CHCl_3$ columns are roughly similar to those found by Sanger (1945) on $CHCl_3$ columns. Buffer *B*, however, probably gives less acid columns than does Sanger's silica gel.

In general, maximum separation is achieved with the least acid columns, although the band rates may then be inconveniently slow. Convenient separations of the slower-moving DNP amino-acids can often be achieved by employing butanol-$CHCl_3$ instead of chloroform as the solvent. A mixture of DNP-glycine and bis-DNP-lysine or bis-DNP-tyrosine does not separate readily on a buffer *B*-$CHCl_3$ column since all three move slowly under these conditions. On a buffer-*B*-3% butanol-$CHCl_3$ column, however, the two latter DNP amino-acids move faster than DNP-glycine and are readily separated from it.

Similarly, a mixture of DNP-threonine, DNP-serine and DNP-aspartic acid can be separated into three bands, emerging from the column in the above order, on buffer *G*-3% butanol-$CHCl_3$ columns or on buffer *B*-17% butanol-$CHCl_3$ columns. With a change in the solvent and buffer in this way, the order in which the DNP amino-acids emerge from the column may be altered. Thus on buffer *A*-$CHCl_3$ columns DNP-glutamic acid emerges ahead of DNP-serine. On more alkaline columns, with butanol-$CHCl_3$ as the mobile phase (on buffer *B*-17% butanol-$CHCl_3$ columns), the *R* value of DNP-serine is greater than that of DNP-glutamic acid. This behaviour may depend on the fact that the ionization of DNP-glutamic acid can involve two carboxyl groups, as opposed to one in the case of DNP-monoamino-monocarboxylic acids. On the gels used by Sanger with 1% butanol-$CHCl_3$ as the mobile phase, DNP-threonine and DNP-serine have faster rates than DNP-glutamic acid and DNP-aspartic acid (Sanger, 1945; Porter & Sanger, 1948).

Propanol-cyclohexane columns

Propanol-*cyclo*hexane (5%) used with buffered columns is a very useful solvent for the separation of DNP-monoamino-monocarboxylic acids. The rates of the DNP amino-acids on these columns are also dependent on the pH of the aqueous phase. Table 2

Table 2. *Band rates (R values) of DNP amino-acids in 5% propanol-cyclohexane*

DNP amino-acid	*R* values (5% propanol-*cyclo*hexane): Buffer *A*	Buffer *B*	Buffer *F*
DNP-leucine	—	0·4	0·5
DNP-valine	Fast	0·15	0·4
DNP-phenylalanine	—	0·2	0·3
DNP-methionine	—	0·06	0·08
DNP-proline	—	—	0·06
DNP-alanine	Fast	0·04	0·10
DNP-tryptophan	—	—	0·11
DNP-glycine	—	0·02	0·02

gives the rates of DNP amino-acids in this solvent. From the band rates quoted by Sanger (1945) for propanol-*cyclo*hexane columns it is evident that buffer *F* gives less acid columns than did Sanger's gels with this solvent. It has been found that buffer *F* columns are suitable for the separation of the majority of the DNP amino-acids of this group. Buffer *B* columns may be used for the separation of the longer-chain DNP amino-acids. The shorter-chain DNP monoamino-acids move rather slowly on buffer *F*-5% propanol-*cyclo*hexane columns, but buffer *G*-10% propanol-*cyclo*hexane columns gives rates suitable for convenient separation.

On buffer *F*-5% propanol-*cyclo*hexane columns DNP-valine can be separated from DNP-alanine, and DNP-alanine from DNP-glycine. DNP-isoleucine and DNP-valine can be readily separated on a buffer *B*-5% propanol-*cyclo*hexane columns. A mixture of DNP-isoleucine and DNP-phenylalanine can also be separated on the latter

column, as can a mixture of DNP-valine and DNP-methionine. A mixture of DNP-isoleucine, DNP-valine, DNP-alanine and DNP-glycine separates into four bands on this column, but the rates of DNP-alanine and DNP-glycine are rather slow.

These columns thus generally affect the separations that can be achieved on ethanol-ligroin columns made according to Sanger (1945). It is noteworthy that both these solvent systems contain alcohols. The type of separation which can be brought about by a given solvent system may therefore be correlated with the chemical character of the solvent. Martin (1948) has previously pointed out the relationship existing between the chemical constitution of the solvent and the extent of separation of amino-acids and their derivatives in partition chromatography.

Ether columns

Buffer *A*-ether columns can be used to free ether extracts of hydrolysates of DNP-proteins and polypeptides from HCl (cf. Sanger, 1945) as all the DNP amino-acids travel fast on these columns. On one occasion when an ether extract of DNP amino-acids from the hydrolysate of a protein treated with 1:2:4-fluorodinitrobenzene was passed through a buffer *G*-ether column to remove HCl, yellow bands, presumably DNP amino-acids, were observed passing down the column. Investigation showed that the DNP amino-acids moved on buffer *B*-ether columns with characteristic rates, which are given in Table 3. These columns are useful for separating the slower moving DNP amino-acids from each other.

Table 3. *Band rates (R values) of DNP amino-acids in ether and* 33 % *ether-ligroin*

DNP amino-acid	Ether, buffer *B*	33 % ether-ligroin, buffer *B*
DNP-leucine	Fast	0·5
DNP-valine	Fast	0·2
DNP-phenylalanine	Fast	0·1
DNP-methionine	1·0	0·1
DNP-proline	0·5	0·04
DNP-alanine	0·5	0·08
DNP-tryptophan	Fast	0·09
DNP-glycine	0·2	0·04
Bis-DNP-lysine	0·6	(Sparingly soluble)
Bis-DNP-tyrosine	0·9	(Sparingly soluble)
DNP-threonine	0·14	—
DNP-serine	0·10	—
DNP-glutamic acid	0·04	—
DNP-aspartic acid	0·01	—

A mixture of DNP-threonine, DNP-serine and DNP-aspartic acid separates into three bands, whilst DNP-threonine or DNP-serine can be separated from DNP-glutamic acid. DNP-glutamic acid separates from DNP-aspartic acid, but the band rates are rather slow for convenient separation. DNP amino-acids which do not separate readily on buffer *B*-$CHCl_3$ columns will often do so on buffer *B*-ether columns. Thus bis-DNP-tyrosine or bis-DNP-lysine will separate from DNP-glycine. The faster moving DNP amino-acids also separate on buffer *B*-ether columns. The band rates are somewhat high, but if more alkaline buffers are employed the bands 'tail' into each other. A mixture of DNP-valine, DNP-alanine and DNP-glycine separates into three bands, and DNP-alanine can be separated from DNP-methionine or DNP-tryptophan.

Ether-ligroin columns

The longer chain DNP monoamino-acids have characteristic rates on buffer *B*-33 % ether-ligroin columns (Table 3). These acids move fast on buffer *B*-ether columns. A mixture of DNP-valine, DNP-alanine and DNP-glycine separates into three bands on a buffer *B*-33 % ether-ligroin column. An extensive investigation has not yet been made, but these columns seem to effect the same separations as buffer *B*-$CHCl_3$ columns.

Columns employing organic solvents as the stationary phase

When 'non-adsorbent' gels were employed it was found that certain of Sanger's columns, which use liquids such as aqueous ethanol or aqueous acetone as the stationary phase, did not separate the appropriate mixtures of DNP amino-acids clearly. Thus with the gels prepared by the present author a mixture of DNP-valine and DNP-phenylalanine was not clearly separated on acetone-*cyclo*hexane columns prepared according to Sanger (1945). When one volume of acetone was mixed with one volume of buffer *G* instead of water and the column then prepared as before, the expected separation was readily obtained.

The separation of DNP-alanine from DNP-proline was also difficult on glycol-benzene columns using 'non-adsorbent' gels. Using the same solvent system both DNP-proline and DNP-alanine moved extremely slowly on a gel which had been prepared by precipitating sodium silicate with acid and washing with buffer (buffer *G*) before drying.

Band rates of acid soluble DNP amino-acids

The band rates of the acid-soluble DNP amino-acids were also found to vary with the pH of the aqueous phase of the column. No systematic investigation of this variation was made, but it was found that columns buffered with buffer *A* gave the desired separations with 66 % methyl ethyl ketone-ether and 30 % butanol-chloroform as the moving phase. These are the solvent systems used by Sanger for these separations.

Separation of mixtures of DNP amino-acids

From the present experiments, and those of Sanger, it is evident that a mixture of DNP amino-acids can be separated into its constituents in a number of different ways, as can a mixture of *N*-acetyl amino-acids (Tristram, 1946). The number of

fractionations necessary to effect a complete separation obviously depends on the number of DNP amino-acids present in the mixture being studied. Buffered columns have been successfully employed by the present author in separating DNP amino-acid mixtures, and the scheme employed differs from that elaborated by Porter & Sanger (1948). The initial fractionation is generally carried out on buffer *B*-ether columns, although buffer-*G*-3% butanol-chloroform columns were employed in the earlier stages of this work. On buffer *B*-ether columns the longer chain DNP monoamino-acids move rapidly down the column followed by DNP-glycine, DNP-threonine and DNP-serine, which can be separated and collected. DNP-glutamic and DNP-aspartic acids form a slow-moving band at the top of the column which splits into its constituents on continued development. Elution of these two bands separately with ether usually required too long a time and it is convenient to elute them together with ether containing a few drops of glacial acetic acid to make the column acid. These DNP amino-acids then run rapidly through the column and, after taking the solution to dryness, can be fractionated on a buffer *A*-chloroform column. The fast-moving first band obtained on buffer *B*-ether columns contains the DNP monoaminomonocarboxylic acids. After taking to dryness, it is refractionated on a buffer *B*-chloroform column. The bands from this fractionation are then further refractionated on buffer *G*-10% propanol-*cyclo*hexane columns for the slower-moving DNP amino-acids and on buffer *B*-5% propanol-*cyclo*hexane columns for the faster-moving DNP amino-acids.

Sanger (1945) observed that partition chromatography on paper was not successful in separating mixtures of DNP amino-acids, owing to 'tailing' of the spots. Phillips & Stephen (1948) claimed some success, particularly with the slower-moving DNP amino-acids, using a two-dimensional technique. Experiments by the present author with a wide variety of solvents led to similar conclusions to those of Sanger (1945), only a partial separation of the different DNP amino-acids being achieved. It is probable that adsorption of the DNP amino-acids on the paper occurs and this effect is superimposed on the partition effects. There is some evidence that adsorption does occur, since when DNP amino-acids were run in distilled water under conditions similar to those of capillary analysis (Rheinboldt, 1925) fairly well-defined spots were formed at some distance from the solvent front. The difference in rate between the different DNP amino-acids was not sufficient to effect separations.

In a few experiments employing partition chromatography, paper previously soaked in buffer solution and then dried was used, but no marked increase in the effectiveness of the separation was achieved. Alumina-treated paper (Datta & Overell, 1949) similarly showed no marked improvement in separation over ordinary paper in a limited number of experiments. Columns of powdered cellulose saturated with buffer solutions effected some degree of separation of DNP amino-acids, but were not as successful as silica-gel columns.

DISCUSSION

Sanger (1945) and Consden *et al.* (1947) considered that the DNP amino-acids are adsorbed to a certain extent on the silica gel. The theory of the partition chromatogram, as initially worked out by Martin & Synge (1941), assumes that the partition coefficient of the solute remains constant with varying concentration, but, as they pointed out, this is seldom the case. If appreciable ionization of the solute, e.g. a DNP amino-acid or *N*-acetyl amino-acid, occurs in the aqueous phase the partition coefficient will vary appreciably with the concentration. As a result the forward boundary of the chromatogram band will become sharper and the back more diffuse, a result commonly observed in ordinary 'adsorption' chromatography where the adsorption follows a Langmuir type isotherm (Tiselius, 1947; Cannan, 1946). Tiselius emphasized that there was hardly any difference in principle between ordinary and partition chromatography. By making the pH of the aqueous phase of the column sufficiently high, the ionization of the DNP amino-acid in the aqueous phase becomes appreciable and the band formed on the chromatogram will be of the adsorption type. The adsorption effects noted by Sanger (1945) and Consden *et al.* (1947) are thus probably due to small amounts of alkali retained by the gel during its preparation. Porter & Sanger (1948) found that adsorbent gels giving low *R* values produced maximum resolution for any given solvent, but gave 'tailing' bands. The present author believes that variations in behaviour between different batches of gel using unbuffered columns are due in large part to variations in the pH of the aqueous phase in contact with the gel. Using a given buffered column, no significant variation has been observed in the rate of a particular DNP amino-acid when different batches of gel are employed. This indicates that variations between different batches of gel, e.g. in the amount of acid or alkali retained, do not cause significant variations in the pH of the aqueous phase of a buffered column. Nevertheless, the band rate of any particular DNP amino-acid varies with the amount of material placed on the column and with the distance the band has travelled down the column. It is possible that some of the variations between batches of gel observed in the separation of *N*-acetyl amino-acids (Tristram, 1946; Martin, 1948) might be eliminated by the use of buffered columns.

The present buffers and solvent systems may not necessarily be the best or the most convenient for separating mixtures of DNP amino-acids, and other schemes of separation using buffered columns could almost certainly be devised. The primary object of the present work was not centred in devising the best analytical method, but in eliminating some of the difficulties encountered using unbuffered columns. The application of these methods will be described in a forthcoming paper.

SUMMARY

The use of buffered silica-gel columns in the partition chromatographic separation of 2:4-dinitrophenyl amino-acids is described.

I am indebted to the Director and Council of the Wool Industries Research Association for permission to publish this paper and to Mr G. R. Lee for assistance with the experimental work.

REFERENCES

Abderhalden, E. & Blumberg, P. (1910). *Hoppe-Seyl. Z.* **65**, 318.
Boon, W. R. (1948). *Analyst*, **73**, 202.
Britton, H. T. S. (1942). *Hydrogen Ions*, **1**, 308 (Table 76). London: Chapman and Hall.
Cannan, R. K. (1946). *Ann. N.Y. Acad. Sci.* **47**, 135.
Consden, R., Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1947). *Biochem. J.* **41**, 596.
Datta, S. P. & Overell, B. G. (1949). *Biochem. J.* **44**, xliii.
Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1943). *Biochem. J.* **37**, 79.
Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1944). *Biochem. J.* **38**, 65.
Martin, A. J. P. (1948). *Ann. N.Y. Acad. Sci.* **49**, 249.
Martin, A. J. P. & Synge, R. L. M. (1941). *Biochem. J.* **35**, 1358.
Phillips, D. M. P. & Stephen, J. M. L. (1948). *Nature, Lond.*, **162**, 152.
Porter, R. R. & Sanger, F. (1948). *Biochem. J.* **42**, 287.
Rheinboldt, H. (1925). In Houben. *Die Methoden der Organischen Chemie*, 3rd ed. **1**, 291. Leipzig: George Thieme.
Sanger, F. (1945). *Biochem. J.* **39**, 507.
Tiselius, A. (1947). *Advanc. protein Chem.* **3**, 67.
Tristram, G. R. (1946). *Biochem. J.* **40**, 721.

The Mechanism of the Action of Notatin

By R. BENTLEY and A. NEUBERGER
National Institute for Medical Research, Hampstead, London, N.W. 3

(*Received* 15 *July* 1949)

Notatin is an enzyme discovered by Müller (1928) in *Aspergillus niger* and in *Penicillium glaucum*, and named by him glucose oxidase. Müller showed that this enzyme catalyses the oxidation of glucose by molecular oxygen to an acidic product which he identified as gluconic acid. Subsequently it was found by Franke & Lorenz (1937), using a more highly purified preparation, that hydrogen peroxide was formed in this reaction; these authors also suggested that the enzyme was a flavoprotein. A more complete purification was achieved by Coulthard, Michaelis, Short, Sykes, Skrimshire, Standfast, Birkinshaw & Raistrick (1942, 1945), who named the enzyme notatin. Independently, Van Bruggen, Reithel, Cain, Katzman, Doisy, Muir, Roberts, Gaby, Homan & Jones (1943) also purified the same enzyme, designated by these authors penicillin B. The experiments of the American workers suggested that the prosthetic group was the alloxazine-adenine dinucleotide. This was definitely proved by Keilin & Hartree (1946, 1948*a*, *b*), who examined the physical and chemical properties of the enzyme in great detail and also determined its specificity range very thoroughly.

The overall reaction catalysed by notatin is generally represented as follows:

$$C_6H_{12}O_6 + O_2 + H_2O = C_6H_{12}O_7 + H_2O_2.$$

This equation does not make clear which is the essential, enzyme-catalysed step in the reaction and, indeed, there has been some discussion as to whether notatin is a dehydrogenase (Franke & Lorenz, 1937; Franke & Deffner, 1939), or an oxidase (Müller, 1936). The latter hypothesis assumes that the enzyme 'activates' molecular oxygen, one of the oxygen atoms combining with the aldehyde form of glucose, whilst the other reacts with water to form hydrogen peroxide. However, the fact that notatin contains alloxazine-adenine dinucleotide suggests that the enzyme is a dehydrogenase. According to this second concept notatin catalyses the transfer of two hydrogen atoms from glucose or from the

hydrated aldehyde form of glucose (Franke & Lorenz, 1937) to molecular oxygen and the latter acts entirely as a hydrogen acceptor.

The present authors (see Bentley, 1948) have found that the oxygen obtained from the decomposition of hydrogen peroxide, by catalase, in water enriched with respect to ^{18}O, contains the normal abundance of ^{18}O. Under these experimental conditions, therefore, there is no exchange of oxygen atoms between hydrogen peroxide and water during the decomposition of the hydrogen peroxide. It thus appeared possible to establish definitely the mechanism of the action of notatin by carrying out the glucose oxidation in water enriched with ^{18}O, and subsequently decomposing the hydrogen peroxide with catalase. If the notatin acts as an oxidase, the oxygen evolved on decomposition of the peroxide should contain half the isotope excess of the water used as a solvent:

$$C_6H_{12}\,{}^{16}O_6 + {}^{16}O_2 + H_2\,{}^{18}O \rightarrow C_6H_{12}\,{}^{16}O_7 + H_2\,{}^{18}O{}^{16}O.$$

If, on the other hand, gaseous oxygen is a hydrogen acceptor in this reaction, the hydrogen peroxide will not contain any excess ^{18}O:

$$C_6H_{12}\,{}^{16}O_6 + {}^{16}O_2 + H_2\,{}^{18}O \rightarrow C_6H_{12}\,{}^{16}O_6\,{}^{18}O + H_2\,{}^{16}O_2.$$

EXPERIMENTAL

Materials

Notatin. This was a highly purified preparation supplied to us by Prof. D. Keilin.

Catalase. Preparatoins were made from horse liver and horse erythrocytes by the methods of Sumner, Dounce & Frampton (1940) and of Bonnichsen (1947), respectively. The haematin content of the catalase solutions was determined according to Keilin & Hartree (1936). The solutions used were between 10^{-7} and 10^{-8} M.

Glucose. In most of the isotope experiments a commercial sample of glucose hydrate was used which consisted almost entirely of the α-form. Pure anhydrous α-glucose was prepared according to Hudson & Dale (1917); it had $[\alpha]_D^{18°} = +101{\cdot}5°$ (5 min.). β-Glucose was prepared by the pyridine method of Behrens (1907) and recrystallized from aqueous ethanol as described by Hudson & Dale (1917); it had $[\alpha]_D^{18°} = +20{\cdot}8°$ (6 min.).

Isotope experiments in $H_2\,{}^{18}O$

These experiments were carried out in a flask (25 ml. capacity) attached through pressure tubing to a 10 ml. gas burette. The flask could be shaken mechanically.

Glucose monohydrate was dissolved in $H_2{}^{18}O$ and the notatin added in a length of melting-point tubing, so that initially it did not dissolve. The apparatus was swept out with normal O_2 and then closed with the burette full of O_2. The flask was agitated so that the notatin dissolved and then shaken mechanically at room temperature, the O_2 uptake being followed in the burette. When there was no further change, the contents of the flask were transferred to one leg of the gas sample tube described by Sprinson & Rittenberg (1948).* The other leg contained a few drops of a catalase solution. The solutions were frozen in an ethanol-CO_2 bath and the tube evacuated to 10^{-3} mm. The tube was then closed by turning the cap, and the solutions allowed to thaw and degas. The contents were again frozen and the evacuation was completed. After thawing, the solutions were mixed to decompose the H_2O_2 and the liberated O_2 admitted to the sample system of the mass spectrometer in the usual way. Water samples were analysed by equilibration with CO_2 in the presence of carbonic anhydrase (Cohn & Urey, 1938; Bentley, 1948).

Possible exchange of oxygen in the decomposition of H_2O_2, *by catalase.* In a large number of control experiments H_2O_2 at various concentrations was decomposed, *in vacuo*, by catalase in the presence of $H_2{}^{18}O$ (1·39 atom % excess ^{18}O). The evolved O_2 was analysed in the mass spectrometer, but was never found to contain a significant excess of ^{18}O. In view of the relatively low degree of enrichment of ^{18}O in the water used, it cannot be concluded that no exchange occurs in the catalytic decomposition of H_2O_2. But it follows that if such an exchange occurs, it must be relatively small and would not affect the interpretation of the experiments reported in this paper. In further control experiments it was shown that there was no isotope exchange between O_2 and $H_2{}^{18}O$ on shaking in the presence of notatin.

Experiments in $^{18}O_2$

Glucose monohydrate dissolved in normal water was placed in one leg of the gas-sample tube of Sprinson & Rittenberg (1948) and the dry notatin in the other leg. The solutions were degassed and the tube evacuated as described above. The tube and contents were kept in an ethanol-CO_2 bath and connected to the anodic part of an apparatus (Bentley, 1949) in which $H_2{}^{18}O$ was electrolysed. When the required quantity of $^{18}O_2$ had been liberated, the tube was closed. The contents were allowed to thaw and the notatin dissolved by mixing. The tube was shaken at room temperature until the reaction was judged complete (3–4 hr.). The contents were frozen and a sample of the $^{18}O_2$ remaining in the tube admitted to the mass spectrometer for isotope analysis. The solution was washed into one leg of the tube and the H_2O_2 decomposed with catalase as described above. The O_2 obtained was analysed in the mass spectrometer.

Potentiometric and polarimetric experiments

Another series of experiments was carried out with the purpose of identifying the primary product of the action of notatin on glucose. In these experiments α- or β-glucose (anhydrous) was dissolved in water (with or without buffer); 0·2 % (w/v) notatin and about 1×10^{-8} M-catalase were then added and the solution made up to volume with water. The solution was then transferred to a conical flask (100 ml.) fitted with inlet and outlet tubes. O_2 was passed in a fairly rapid stream through the inlet tube whilst the flask was shaken mechanically. The temperature in all experiments was $30 \pm 0{\cdot}2°$, unless otherwise stated.

Polarimetric measurements were made with a 2 dm. tube at 19–20°. The pH was measured with a glass electrode using a Cambridge Instrument Co. portable pH meter.

* One of us (R. Bentley) is very much indebted to Dr Rittenberg for a description of this tube in advance of publication.

Enzymic oxidation was followed either by estimating the remaining glucose by titration with hypoiodite (Willstätter & Schudel, 1918) or by titrating alkalimetrically the total gluconic acid formed.

Total gluconic acid. As will be shown later, a lactone of gluconic acid is the first product of the enzymic reaction. It was therefore necessary, in order to estimate the combined and free gluconic acid and lactone, to boil the solution after each addition of alkali. The latter was added until the pink colour of phenolphthalein persisted for at least 1 min. The blank values obtained by titrating samples withdrawn at zero time were generally very small unless appreciable amounts of buffer were used. The values obtained by the two methods agreed generally within 3%. However, the alkalimetric method was found to be more reliable and figures for oxidation are based only on the total gluconic acid values, unless otherwise stated.

Free gluconic acid. This was estimated by titrating a sample with 0·05 N-NH_3 at 1–3° to a pH of about 6·0–6·5, using B.D.H. Universal Indicator. The results obtained by this method were only accurate within about 5–10%, since hydrolysis of the lactone during the titration could not be completely avoided.

RESULTS

Isotope experiments

Experiments with $H_2{}^{18}O$. Table 1 shows that there is no detectable isotope excess in the hydrogen peroxide produced in the notatin reaction, if ordinary oxygen and isotopically enriched water are used. The figures for oxygen uptake indicate that only about 80–90% of the total glucose has been oxidized. This is caused by the accumulation of hydrogen peroxide owing to the absence of catalase in the first phase of the experiment, and also by the absence of buffer.

Experiments with $^{18}O_2$. If the oxidation of glucose by notatin is carried out with isotopically enriched oxygen and ordinary water, the oxygen evolved from the hydrogen peroxide has the same isotope composition as the gaseous oxygen used (Table 2). Both sets of experiments prove conclusively that the oxygen atoms of the hydrogen peroxide produced in the notatin reaction, are not derived from the solvent, but from the oxygen of the gas phase.

The relative rates of oxidation of α- and β-glucose

Fig. 1 shows the results obtained with α- and β-glucose respectively. In these experiments the glucose was added last to the solution in order to minimize the effect of mutarotation of the glucose. It appears that the enzyme acts on both forms, although the β-form is oxidized about 1·3 times more quickly than the α-form. It cannot be assumed that α-glucose is first converted to β-glucose by spontaneous mutarotation and then attacked by the enzyme, since the rate of mutarotation of glucose under the conditions used is too slow, compared with that of the enzymic oxidation. Moreover, the shape of the curve does not indicate that the rate of oxidation of α-glucose increases with time. It was considered possible that the enzyme catalyses the mutarotation of glucose. This was tested in the following manner:

Anhydrous α-glucose (0·54 g.) was dissolved in 25 ml. of water which had been boiled to remove O_2 and then quickly cooled to 20°; to 10 ml. of this solution was added 0·5 ml. of 0·2% notatin and 7·5 ml. of water, whilst the control was made up to the same volume with water. Both samples

Table 1. *Atom % excess* ^{18}O *in hydrogen peroxide formed on oxidation of glucose by notatin with* $^{18}O_2$ *in* $H_2{}^{18}O$

(Experimental error of isotope analysis for ^{18}O in samples of normal or nearly normal abundance = ±0·003 atom %.)

Exp. no.	Glucose monohydrate (mg.)	Notatin (mg.)	$H_2{}^{18}O$ (ml.)	Atom % excess ^{18}O in water	Duration of exp. (hr.)	O_2 uptake at N.T.P. (ml.)	Atom % excess ^{18}O in H_2O_2
1	75	0·19	1·0	1·01	2·5	6	0·002
2	100	0·5	2·0	1·01	3·0	10	0·003
3	100	0·5	2·0	1·01	4·0	9·5	0·003

Table 2. *Atom %* ^{18}O *in hydrogen peroxide formed on oxidation of glucose by notatin with* $^{18}O_2$ *in* $H_2{}^{16}O$

(Experimental error of isotope analysis in samples of about 0·6 atom % excess = ±0·005%. α-Glucose monohydrate was used in Exps. 1 and 2 and anhydrous β-glucose in Exp. 3.)

Exp. no.	Glucose (mg.)	Notatin (mg.)	Water (ml.)	$^{18}O_2$ evolved (calc.) at N.T.P. (ml.)	Temp. (°)	Duration of exp. (hr.)	Atom % excess ^{18}O of: Electrolytic oxygen	Oxygen remaining at end of incubation	Oxygen from peroxide decomposition
1	100	0·5	1	8·6	18	3·5	0·603	0·563*	0·563
2	100	0·5	1	10·2	18	3·75	0·591	0·593	0·587
3	100	0·5	1	8·6	37	1	—	0·579	0·580

* This dilution was due to an air bubble accidentally admitted during electrolysis. The presence of air was confirmed by an ion beam in the spectrometer at mass 40, showing the presence of argon.

were transferred to polarimeter tubes and the changes of optical rotation observed for 4 hr. The following $[\alpha]_D$ values were obtained for the control solution: +92·5° (22·5 min.); +87·5° (31·5 min.); +68·5° (74·5 min.); +55·4° (300 min.). The sample containing notatin had the following $[\alpha]_D$ values: +76·2° (23 min.); +69·1° (32 min.); 55·5° (75 min.); +51·7° (300 min.). There thus seemed to be a definite difference between the two solutions. However, it was found

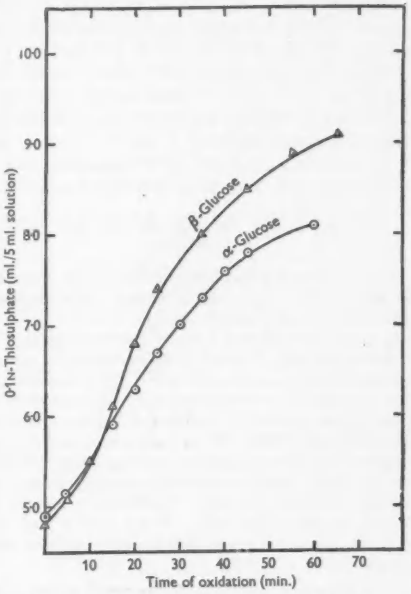


Fig. 1. The relative rates of oxidation of α- and β-glucose by notatin. Anhydrous α- or β-glucose (600 mg.) was added to 60 ml. solution containing M-acetate buffer pH 5·7 (4 ml.), catalase (0·5 ml.) and notatin (0·4 mg.). Oxidation was at 22° for the times indicated by the abscissae; 0·1 N-I_2 (10 ml.) and 0·1 N-NaOH (15 ml.) were then added to 5 ml. samples. After standing for 5 min., the I_2 not used up was titrated with 0·1 N-$Na_2S_2O_3$ after the addition of 2·5 ml. 5 N-H_2SO_4.

that the notatin had brought about the oxidation of about 3–4% of the glucose. The specific rotations of gluconic acid and the δ-lactone are smaller than that of α-glucose and this will account for a small part of the difference. Moreover, the formation of free gluconic acid and the consequent change of pH is bound to affect the non-enzymic mutarotation of glucose. However, the slight oxidation found cannot explain the results obtained.

There is thus suggestive, but no conclusive evidence that notatin catalyses the mutarotation of glucose and that α-glucose is converted enzymically to β-glucose before oxidation can take place.

The nature of the primary product of oxidation

Comparison between the development of acidity and the disappearance of glucose. Gluconic acid is a moderately strong acid. Thus gluconic acid solutions prepared by treatment of calcium D-gluconate hydrate with the calculated amount of oxalic acid had the following pH values: 0·001 M, 3·18; 0·01 M, 2·71; 0·1 M, 2·25. A 0·1 M solution half neutralized with 0·1 N-NaOH had a pH of 3·66. The pK of gluconic acid must therefore be about 3·6–3·7.* It is to be expected therefore that the formation of a small amount of free gluconic acid will, in the absence of buffering, reduce the pH to below 3·5. The pH optimum for notatin is about 5·6 (Keilin & Hartree, 1948*a*) and the rate of oxidation falls off rapidly from pH 4 to 3·0 to become quite small at higher acidities. Müller (1928) had already found that a decrease of pH and increase of base binding occurs in the notatin reaction and this retards the action of the enzyme. It was thought, therefore, that gluconic acid itself is the first product of this oxidation. However, it can be shown that the development of acidity is not part of the oxidation process, but occurs later and is almost certainly not catalysed by the enzyme. This is clearly shown by the following experiments.

Oxidation with a large amount of enzyme, but without buffer. β-Glucose (80 mg.) was added to a solution (total vol. 15 ml.) containing 2·4 ml. 0·2% notatin and 0·5 ml. catalase. Oxidation was allowed to proceed for 8 min.; by that time 84% of the glucose had been oxidized. The initial pH was 5·4 and at the end of the oxidation 3·55. The solution was now cooled to 20° and left to stand under N_2. The pH changes were as follows: 15 min. after the addition of enzyme, 3·15; 20 min., 2·99; 40 min., 2·75; 60 min., 2·55. There was no further oxidation during the second part of the experiment.

Oxidation in the presence of a small amount of buffer. In this experiment the amount of base in the acetate buffer was equivalent to 10% of the glucose used. β-Glucose (0·54 g.) was dissolved in a solution (total vol. 30 ml.) containing 3 ml. 0·12 N-acetic acid, 3 ml. 0·1 N-NaOH, 0·3 ml. catalase solution and 3 ml. 0·2% notatin. Oxidation was allowed to proceed for 0·5 hr. During that time the pH had fallen from 5·58 to 4·29 and 94% of the glucose had been oxidized. The solution was again cooled to 20° and the pH changes noted for the following 15 hr. During the second part of the experiment a further 3% of the glucose was oxidized. The pH changes were as follows. Times (in hr.) from the beginning of the experiment are given in brackets: 4·15, (0·8); 4·00, (1·1); 3·88, (1·5); 3·78, (1·75); 3·69, (2·1); 3·55, (2·7); 3·38, (4·0); 3·27, (5·0); 3·08, (8·0); 3·02, (16·0).

In this experiment the pH did not fall below 3·0 owing to the buffering effect of the gluconate ion; sodium acetate and gluconic acid must have reacted to give acetic acid and sodium gluconate.

* May, Weisberg & Herrick (1929) found a value of 3·78 for the pK of gluconic acid.

Measurement of free and total gluconic acid. Table 3 shows that the primary product is a substance which does not titrate as an acid under the conditions used for the estimation of free gluconic acid, but can be estimated under the more vigorous conditions employed for 'total' gluconic acid. The most reasonable assumption is that this substance is a lactone. The relative proportion of free gluconic acid is small in the beginning of the oxidation, but increases rapidly, after most of the oxidation is finished. This is in accordance with the pH measurements reported above. The converse, of course, applies to the lactone which is measured by the difference between total and free gluconic acid.

Table 3. *Comparison of the rate of formation of free and total gluconic acid during the oxidation of glucose by notatin*

(α-Glucose (0·540 g.) was dissolved in 60 ml. water containing 0·41 mg. notatin and 0·5 ml. catalase. Oxidation proceeded at 24°. 3 ml. samples were withdrawn at suitable intervals and titrated for free and total gluconic acid as described in the text. The very small blank values were subtracted.)

Time after addition of glucose (min.)	Percentage of glucose originally present converted into		Ratio of total/free gluconic acid
	(*a*) Free gluconic acid	(*b*) Total gluconic acid	
10	0·5	10·9	21·8
20	1·0	17·9	17·9
30	1·6	24·0	15·0
60	5·1	42·4	8·3
90	9·3	52·5	5·6
128	19·0	59·3	3·2
160	35·0	75·1	2·1
1080	84·2	100·1	1·18

Polarimetric measurements

The experiments so far reported indicated that notatin converts glucose to a lactone or some other neutral substance which is slowly converted to gluconic acid. A more definite identification of this primary product was achieved by following the changes in optical rotation. δ-Gluconolactone has an initial $[\alpha]_D$ of +66·2° (Nef, 1914; Isbell & Frush, 1933) which falls over 24 hr. to +8·8°, whilst γ-gluconolactone reaches an equilibrium value much more slowly. Fig. 2 shows that the primary product of the notatin reaction resembles δ-gluconolactone very closely; the slight differences between the mutarotation curves found here and those reported by Nef (1914) or by Isbell & Hudson (1932) are caused by the fact that in experiments with pure δ-lactone all the lactone is present at zero time, while in the enzymic experiments it is being formed from glucose during the first 20–30 min. of the experiment. The small amount of glucose left at the end of the experiment (6·5 and 7% respectively) explains the relatively high equilibrium figure after 24 hr., which was +12·1° instead of +8·8° found by the other workers.

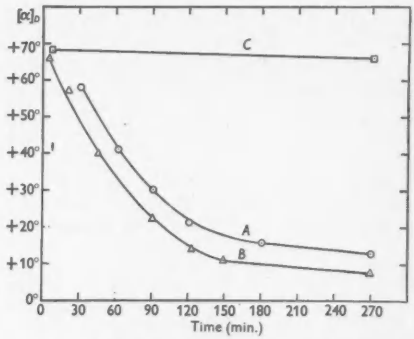


Fig. 2. Comparison of the mutarotation of the product of the oxidation of β-glucose by notatin with the mutarotation of δ- and γ-gluconolactone. Curve *A* gives the $[\alpha]_D$ values (calculated in terms of gluconolactone) of the oxidation product. Curve *B* is the mutarotation curve of δ-gluconolactone, and curve *C* is that of γ-gluconolactone. The data for the two lactones were obtained from the figures given by Nef (1914), Isbell & Hudson (1932) and Isbell & Frush (1933). The oxidation was carried out as follows: Anhydrous β-glucose (600 mg.) was added to 30 ml. of a solution containing notatin (4·8 mg.) and catalase solution (0·4 ml.). Oxidation proceeded for 25 min. at 30°, when 93% of the glucose had been oxidized. The solution was then filtered and the rotation measured.

DISCUSSION

The isotope experiments prove conclusively that notatin catalyses the transfer of hydrogen from glucose to the oxygen of the gas phase. The fact that there was no isotope exchange between $H_2{}^{18}O$ and $^{16}O_2$ on shaking with notatin is confirmatory evidence that this enzyme does not produce a direct 'activation' of molecular oxygen, similar to the 'activation' of hydrogen produced by the enzyme system of *Proteus vulgaris* (Hoberman & Rittenberg, 1943).

At first, the most likely substrate of notatin appeared to be the hydrate of the aldehyde form of glucose—which could be dehydrogenated to the free gluconic acid in the manner suggested by Wieland (1932). However, Goto & Titani (1941) have shown that at room temperature, but not at 100°, the rate of exchange of glucose with $H_2{}^{18}O$ is very slow and that mutarotation takes place without exchange of oxygen. Since reversible hydration of glucose necessarily involves oxygen exchange on C_1, it follows that such an uncatalysed hydration is slow at room temperature. Thus, if the actual substrate of dehydrogenation were a glucose aldehyde hydrate,

it would have to be assumed that notatin, in addition to its oxidizing action, also catalyses the conversion of ring forms of glucose to the open aldehyde form and/or the hydration of the aldehyde. Such a rather complicated hypothesis becomes unnecessary in view of the experiments reported now.

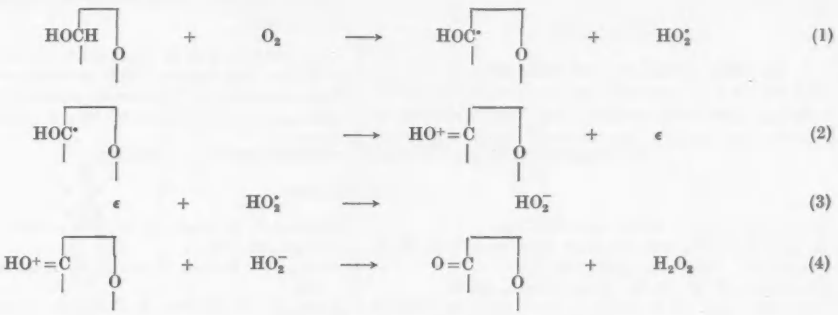
The fact that the development of acidity is not directly associated with the enzymic process and can be made to take place almost entirely after the oxidation has stopped, shows that the primary product is not gluconic acid. This is also shown by the comparison of the formation of 'free' and 'total' gluconic acid. From the polarimetric experiments it can be deduced that the neutral primary product is δ-gluconolactone. It thus follows that the substrate of notatin is glucopyranose and not the free aldehyde, or its hydrate, or one of the two glucofuranoses. If the aldehyde were the substrate, the primary product would be gluconic acid, whilst the oxidation of a furanose would have yielded the γ-lactone.

The results reported here may suggest that notatin acts on both α- and β-glucopyranose, though the rate is definitely faster with the β-isomeride. However, suggestive evidence was obtained that notatin catalyses the mutarotation of α-glucose, and it is very probable that mutarotation catalysed by notatin precedes oxidation.* Keilin & Hartree (1948*a*) have recently examined the substrate specificity of notatin in some detail. They have found that mannose and xylose are oxidized at about 1% of the rate found for glucose, and galactose is also attacked, albeit more slowly. It is of interest that the action of bromine on glucose in a buffered, weakly acidic medium also yields δ-gluconolactone and not gluconic acid (Isbell & Hudson, 1932; Isbell, 1932). However, in this reaction the rate is about 35 times faster with β- than with α-glucose (Isbell & Pigman, 1933). Oxidation with hypochlorite, on the other hand, is faster with α- than with β-glucose (Jeannes & Isbell, 1941).

* Keilin & Hartree (private communication) have independently discovered the effect of notatin on the mutarotation of α-glucose and have made this problem the subject of an extensive investigation.

Detailed mechanism of the hydrogen transfer

In the reaction under discussion, two protons and two electrons are transferred from glucopyranose through the flavoprotein to molecular oxygen. This could occur in two successive steps, each involving the transfer of one electron; this mechanism may be pictured as follows (leaving out the intermediate reactions involving the prosthetic group of the enzyme):



In the first step the glucopyranose loses a proton and an electron to give two free radicals, $HO_2^•$ and a radical derived from glucose; the latter then loses a second electron to form a carbonium ion which is stabilized by resonance with an oxonium ion. A prototropic reaction between the oxonium ion and the peroxide anion then completes the oxidation. Riboflavin and flavoproteins can exist in the form of fairly stable free radicals (Haas, 1937; Michaelis & Schwarzenbach, 1938), and preliminary experiments carried out here appear to show that notatin can catalyse the reaction between titanous chloride and iodine, presumably by the mechanism suggested by Shaffer (1933, 1936). It can therefore be assumed that notatin may be an intermediate in reactions involving the transfer of one electron by being itself transformed into a free radical. However, there is no evidence for even the transient existence of a free radical derived from glucose, and the known facts are equally compatible with the assumption that the oxidation of glucose by notatin occurs by an ionic mechanism.

According to this concept the essential step in the reaction is the transfer of a hydride ion, i.e. a proton with two electrons, from the glucopyranose to mole-

cular oxygen, and thus the reactions (1), (2) and (3) are telescoped into one:

(5)

This is followed by reaction (4) as above. The shift of a hydride ion has been postulated as the mechanism of other redox reactions such as a Cannizzaro reaction (viz. Hammett, 1940). No facts are available which would enable us to decide which of the two interpretations is correct. In any case, the distinction may be somewhat unreal, since the ionic mechanism may be considered to represent an extreme case of the free radical mechanism in which reactions (2) and (3) are infinitely fast compared with reaction (1).

SUMMARY

1. By using water and molecular oxygen labelled with ^{18}O it is shown that the oxygen atoms of the hydrogen peroxide produced in the oxidation of glucose by notatin are derived from molecular oxygen. It is concluded that notatin catalyses the transfer of hydrogen from glucose to oxygen.

2. A comparison of the rate of oxidation of glucose by notatin and the development of acidity shows that the first product of the enzymic action is a neutral substance. Polarimetric measurements indicate that this is δ-gluconolactone which changes by a non-enzymic reaction into gluconic acid.

3. The mechanism of the transfer of two hydrogen atoms of glucose to oxygen is discussed. Two alternative interpretations are proposed, one involving a free radical mechanism and the other a simultaneous transfer of two electrons as the rate-determining step.

The authors wish to thank Prof. D. Keilin, F.R.S., for putting at their disposal highly purified notatin, Miss M. G. French and Miss B. Wiltshire for assistance in the chemical work and Mr G. Dickinson for help with the mass spectrometer.

REFERENCES

Behrens, R. (1907). *Liebigs Ann.* **353**, 106.
Bentley, R. (1948). *Cold Spr. Harb. Sym. quant. Biol.* **13**, 11.
Bentley, R. (1949). *Biochem. J.* **45**, 591.
Bonnichsen, R. K. (1947). *Arch. Biochem.* **12**, 83.
Cohn, M. & Urey, H. C. (1938). *J. Amer. chem. Soc.* **60**, 679.
Coulthard, C. E., Michaelis, R., Short, W. F., Sykes, G., Skrimshire, G. E. H., Standfast, A. F. B., Birkinshaw, J. H. & Raistrick, H. (1942). *Nature, Lond.*, **150**, 634.
Coulthard, C. E., Michaelis, R., Short, W. F., Sykes, G., Skrimshire, G. E. H., Standfast, A. F. B., Birkinshaw, J. H. & Raistrick, H. (1945). *Biochem. J.* **39**, 24.
Franke, W. & Deffner, M. (1939). *Liebigs Ann.* **541**, 117.
Franke, W. & Lorenz, F. (1937). *Liebigs Ann.* **531**, 1.
Goto, K. & Titani, T. (1941). *Bull. chem. Soc. Japan.* **16**, 403.
Haas, E. (1937). *Biochem. Z.* **290**, 291.
Hammett, L. P. (1940). *Physical Organic Chemistry*, 1st ed. New York–London: McGraw-Hill.
Hoberman, H. D. & Rittenberg, D. (1943). *J. biol. Chem.* **147**, 211.
Hudson, C. S. & Dale, J. K. (1917). *J. Amer. chem. Soc.* **39**, 322.
Isbell, H. S. (1932). *Bur. Stand. J. Res., Wash.*, **8**, 615.
Isbell, H. S. & Frush, H. L. (1933). *Bur. Stand. J. Res., Wash.*, **11**, 649.
Isbell, H. S. & Hudson, C. S. (1932). *Bur. Stand. J. Res., Wash.*, **8**, 327.
Isbell, H. S. & Pigman, W. W. (1933). *Bur. Stand. J. Res., Wash.*, **10**, 337.
Jeannes, A. & Isbell, H. S. (1941). *Bur. Stand. J. Res., Wash.*, **27**, 125.
Keilin, D. & Hartree, E. F. (1936). *Proc. Roy. Soc.* B, **121**, 173.
Keilin, D. & Hartree, E. F. (1946). *Nature, Lond.*, **157**, 801.
Keilin, D. & Hartree, E. F. (1948*a*). *Biochem. J.* **42**, 221.
Keilin, D. & Hartree, E. F. (1948*b*). *Biochem. J.* **42**, 230.
May, O. E., Weisberg, S. M. & Herrick, H. T. (1929). *J. Wash. Acad. Sci.* **19**, 443.
Michaelis, L. & Schwarzenbach, C. (1938). *J. biol. Chem.* **123**, 527.
Müller, D. (1928). *Biochem. Z.* **199**, 236.
Müller, D. (1936). *Ergebn. Enzymforsch.* **5**, 259.
Nef, J. V. (1914). *Liebigs Ann.* **403**, 204.
Shaffer, P. A. (1933). *J. Amer. chem. Soc.* **55**, 2169.
Shaffer, P. A. (1936). *J. phys. Chem.* **40**, 1021.
Sprinson, D. B. & Rittenberg, D. (1948). *Nav. med. Bull., Wash.*, Suppl. March–April, p. 82.
Sumner, J. B., Dounce, A. L. & Frampton, V. L. (1940). *J. biol. Chem.* **136**, 343.
Van Bruggen, J. T., Reithel, F. J., Cain, C. K., Katzman, P. A., Doisy, E. A., Muir, R. D., Roberts, E. C., Gaby, W. L., Homan, D. M. & Jones, L. R. (1943). *J. biol. Chem.* **148**, 365.
Wieland, H. (1932). *On the Mechanism of Oxidation*, 1st ed. New Haven: Yale University Press.
Willstätter, R. & Schudel, G. (1918). *Ber. dtsch. chem. Ges.* **51**, 780.

An Apparatus for the Micro-electrolysis of Water

By R. BENTLEY, *National Institute for Medical Research, London, N.W. 3*

(*Received* 15 *July* 1949)

In tracer work using D and ^{18}O it is often desirable to be able to decompose small amounts of water. This is particularly the case with ^{18}O, where rather limited amounts of ^{18}O are available as $H_2{}^{18}O$. In a study of the mechanism of the action of notatin (Bentley & Neuberger, 1949) the isotope was required as oxygen gas; an apparatus was devised in which quantities of the order of 1·5 ml. of water may be satisfactorily electrolysed and the oxygen collected in a vacuum system.

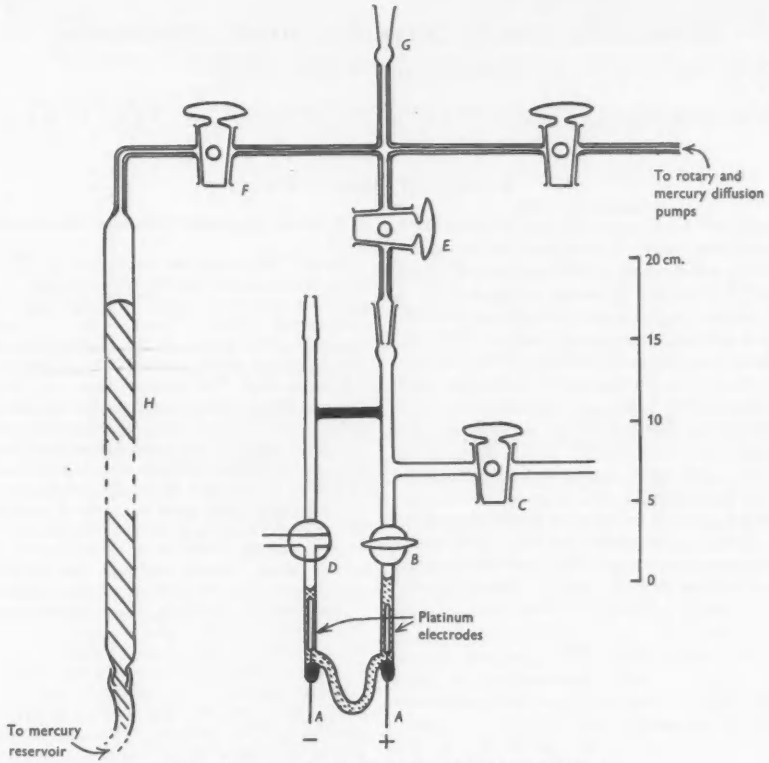


Fig. 1. An apparatus for the micro-electrolysis of water.

EXPERIMENTAL

The apparatus (Fig. 1) was constructed in pyrex glass: 6 mm. tubing was used for the cell, and 3 mm. capillary for the rest of the vacuum system. The electrodes consisted each of a roll (1 sq.in.) of platinum foil spot-welded via a short length of platinum to the tungsten wires *A* (1 mm. diam.) sealed into each arm of the U-tube. In operation, degassed $H_2{}^{18}O$ (about 1·5 ml.) containing a little Na_2SO_4 was placed in the U-tube, and the apparatus evacuated with stopcocks *B* and *C* closed. (All stopcocks were greased with Apiezon vacuum grease, grade L.) By cautious manipulation of stopcock *B* the water was allowed to rise just to the barrel. Stopcock *B* was then closed, and approximately 200 V. d.c. applied to the electrodes, so that the current flowing was about 0·1 amp. Hydrogen produced in the cathode limb was allowed to escape, or could be collected separately depending on the position of the 3-way stopcock *D*. The oxygen evolved was collected under the barrel of stopcock *B*, and as the level of water in this limb diminished, stopcock *B* was cautiously opened so that the oxygen

escaped into the vacuum system and the water level was restored. It was necessary to cool the cell during electrolysis and for this purpose it was wound with heavy-gauge copper wire, the ends of which dipped into an ethanol-CO_2 bath. As this cooling was still insufficient, the cold ethanol was splashed over the cell with a small brush. With a current of 0·11 amp., 1·6 ml. of $^{18}O_2$ at N.T.P. were evolved in 5 min.

When electrolysis was complete, the oxygen was expanded into the Toepler pump (*H*) with stopcocks *E* and *F* opened. With stopcock *E* closed it was then pumped into a previously evacuated apparatus, attached to the vacuum line through the standard joint *G* (B10). With water of 0·575 atom % excess ^{18}O in the cell (determined by equilibration with CO_2 and carbonic anhydrase as described by Bentley, 1948), oxygen gas of 0·579 atom % excess ^{18}O was obtained.

SUMMARY

An apparatus is described in which quantities of the order of 1·5 ml. of water may be electrolysed, and the evolved oxygen (or hydrogen) collected in a vacuum system.

The author is indebted to Miss M. G. French for experimental assistance and to Mr G. Dickinson for assistance with mass spectrometric analyses.

REFERENCES

Bentley, R. (1948). *Cold Spr. Harb. Sym. quant. Biol.* **13**, 11.

Bentley, R. & Neuberger, A. (1949). *Biochem. J.* **45**, 584.

Growth Factors for *Corynebacterium Diphtheriae*

4. PREPARATION FROM YEAST

By F. W. CHATTAWAY, DORIS E. DOLBY, D. A. HALL and FRANK C. HAPPOLD
Department of Biochemistry, University of Leeds

(*Received* 15 *February* 1949)

In an earlier paper, Chattaway, Happold & Sandford (1944) showed that certain gravis (and intermedius) strains of *Corynebacterium diphtheriae* required an unidentified growth factor when cultivated on a medium of known composition containing pimelic, nicotinic and pantothenic acids (Mueller, 1937*a*, *b*; Evans, Handley & Happold, 1939*a*, *b*). This factor, present in liver, was shown to be dialysable and resistant to proteolytic enzymes. Its properties were consistent with the presence in its molecule of both acid and basic groups probably in the form of an α-amino-acid component, since the activity was destroyed by ninhydrin.

The factor appeared to have no direct connexion with any of the unidentified growth factors for *Lactobacillus casei*, cf. Dolby, Happold & Sandford (1944), but showed some similarity to strepogenin, which was prepared by Woolley (1941) from various purified proteins, and is required by certain strains of *Streptococcus haemolyticus*. The present paper reports the separation and concentration from yeast of a number of factors similar in many respects to the liver factor of Chattaway *et al.* (1944).

EXPERIMENTAL

Method of microbiological assay

Organism. The strain of *C. diphtheriae* used for assessing the potency of preparations was a gravis strain, subtype Dundee (Barton). In all cases the growth was observed in $6 \times \frac{5}{8}$ in. test tubes containing the medium outlined below.

Inocula. The strain was maintained on Löffler slopes in the refrigerator and subcultures were made on blood-agar plates. A sufficient amount of an 18 hr. subculture was introduced into 10 ml. of sterile water to produce a faint turbidity at the surface. This suspension was dispersed by shaking and one drop (approx. 0·03 ml.) used for inoculation.

Basal medium. The basal medium employed, shown in Table 1, differed from that described by Chattaway *et al.* (1944). Certain amino-acids hitherto omitted were included in order to bring its composition more nearly into line with a casein-hydrolysate medium on which the organism grows profusely. In the early experiments it was found that test cultures occasionally grew in the basal medium. This was traced to an impurity in the aspartic acid, which was then omitted without alteration in the response of the organism to the factor. Pimelic acid was also omitted since the organism was provided with preformed biotin.

Determination of growth. The organism used grows exclusively as a pellicle under the conditions of test, a fact which enables a visual assay of growth to be made, and which precludes all other methods with the exception of a determination of the N_2 content of a whole culture. This latter method was discarded since an adequate expression of growth could be made in terms of arbitrary degrees of pellicle formation. Ten degrees of growth were recognized, ranging from one given a numerical value of 4, corresponding to a heavy pellicle over the surface of the medium, through 3, 2, 1·5 and 1, at which value the whole surface of the medium was covered with a thin film of growth, down to degrees of growth given the numerical values 0·5, 0·2, 0·1, 0·05 and 0·02, the last being a just discernible trace. Growth was determined after 72 hr. incubation at 37°. This method

Table 1. *Basal medium for the growth of* Corynebacterium diphtheriae (*Dundee*)

(Quantities for 1 l.)

	mg.		μg.
Glycine	100	Biotin	1
DL-Alanine	400	*p*-Aminobenzoic acid	50
L-Valine	700	Riboflavin	100
L-Leucine	500	Nicotinic acid	200
L-Isoleucine	100	Calcium pantothenate	100
L-Proline	400	Pyridoxine	600
L-Hydroxyproline	20		
DL-Phenylalanine	200	2M-sodium lactate (from B.D.H. A.R. lactic acid)	20 ml.
DL-Aspartic acid	400		
DL-Glutamic acid hydrochloride	2200	$Na_2HPO_4.12H_2O$	1 g.
DL-Serine	200	K_2HPO_4	0·5 g.
L-Tyrosine	300	$FeSO_4.7H_2O$	160 μg.
DL-Methionine	100	NaCl	9 g.
D-Arginine	400	$MgSO_4.7H_2O$	0·7 g.
L-Histidine (base)	400		
L-Lysine hydrochloride	600	Salts solution:	1·0 ml.
L-Tryptophan	400	$CuSO_4.5H_2O$ 0·05 g.	
L-Cystine	800	$ZnSO_4.7H_2O$ 0·04 g. } in 50 ml. water	
DL-Threonine	50	$MnCl_2.4H_2O$ 0·02 g.	

The medium was adjusted to pH 7·4 and 0·05 ml. of 0·5% $CaCl_2$/5 ml. of medium added aseptically after autoclaving.

Table 2. *The activity of yeast fractions as growth-promoting substances for* Corynebacterium diphtheriae

Fraction	x=	Concentration of additions (10^{-x} ml./ml. medium)									
		1	2	3	4	5	6	7	8	9	10
		(Activity numbers)									
Hydrolysate after removal of HCl (dry weight 250 mg./ml.)		17	100	17	1	0	0	—	—	—	—
Filtrate from norite		67	100	33	17	7	3	0	—	—	—
Filtrate after removal of inorganic material		33	100	100	67	50	33	7	0	—	—
After removal of lead as phosphate		0	17	67	100	67	33	17	1	0	—
Filtrate from phenylhydrazone containing some phenylhydrazine		0	0	100	100	67	50	33	17	7	—
Final extract after purification from phenylhydrazine and acetic acid (dry weight 170 mg./ml.)		33	67	100	100	100	100	100	67	33	17

has proved useful since several factors are capable of causing variation in the maximum growth obtained in a test and an approximate numerical expression of the results permits the values obtained in each series of tests to be expressed as a percentage of the maximum growth obtained at that time. These values, termed activity numbers, can then be compared in tests carried out at different times.

Preparation of active concentrate

Choice of material. Previous work had been carried out on liver residues from the preparation of the antipernicious anaemia factor (the factory treatment of which was unknown to us); later samples were found to be entirely without activity presumably due to some alteration in technique. Other sources, therefore, had to be explored. Both casein hydrolysates and yeast hydrolysates contain sufficient factor for growth of the organism, but it was decided to use yeast as large quantities were available.

Fresh brewer's yeast was usually employed, but one sample of dried baker's yeast showed similar activity when worked up in the same manner. Brewer's yeast was obtained within 24 hr. of draining and was not steamed; longer periods of standing resulted in autolysis and loss of activity. The active material is an essential growth factor for *Strep. faecalis* R and *Lb. casei* when these organisms are grown on amino-acid media. Material has also been prepared from human urine which is active for the above strain of *C. diphtheriae*.

Preparation of active extracts. Fresh brewer's yeast (2 kg.) was made into a slurry with 8 l. of 2N-HCl, boiled rapidly for 1·5 hr., filtered, and the residue washed with 1 l. of water. PbO was added to the combined filtrate and washings to bring it to pH 2·8–3·0, the precipitate removed and the filtrate and washings shaken with charcoal (norite 200 g./l.) for 2 hr., and filtered. The norite was washed with water, the washings added to the filtrate and the pH adjusted to 6·5 with Na_3PO_4 in saturated solution. The filtrate, after the removal of lead phosphate, was concentrated to about 4 l. using capryl alcohol as an antifoam agent. To remove sugar impurities (mainly mannose) hydrated sodium acetate (2 kg.) and phenylhydrazine hydrochloride (200 g.) were added in 4 l. of water, and the phenylhydrazones filtered off 18 hr. later. Most of the excess phenylhydrazine was removed from the filtrate in the usual way with benzaldehyde (25 g.), the remainder by nine extractions with 1/3 vol. of ether. Acetic acid was removed by acidification with H_2SO_4 to pH 3, followed by repeated extraction with ether. The solution was then frozen repeatedly, and the successive crops of sodium sulphate crystals removed. The solution was concentrated *in*

vacuo below 40°, to about 1·2 l., treated with ethanol to a concentration of 40% (v/v), and the pH brought to 6·5 with baryta. The solution (*Y* 47) after removal of $BaSO_4$ had a dry weight of 170 mg./ml. and was active at $1·7 \times 10^{-5}$ μg./ml. of medium. Table 2 gives the activities of fractions at various stages throughout the concentration.

Properties of yeast concentrate Y 47

Analytical figures are shown in Table 3. The preparation contained an appreciable quantity of free amino-acids, and also peptides, indicated by a positive biuret reaction. No histidine, tyrosine, arginine, tryptophan or free amines were present. Application of the reaction of Elson & Morgan (1933) showed the presence of material akin to amino sugars but not identical with glucosamine.

Table 3. *Analyses of yeast extract Y 47*

(Dry weight of *Y* 47 = 170 mg./ml.)

Material determined	Content (mg./100 mg. *Y* 47)
Total N (Kjeldahl)	6·26
NH_2-N	2·33
α-NH_2-N (ninhydrin)	0·92
S	Nil
Total P	0·39
Inorganic P	0·03
Carbohydrate as glucose (Hagedorn & Jensen)	53·2
Carbohydrate as glucose (Somogyi)	33·5

Inactivation. (*a*) *Hydrolysis.* Prolonged hydrolysis with 6N-HCl considerably reduced activity. Acid hydrolysis did not affect the α-amino N value appreciably.

(*b*) *Ninhydrin.* Refluxing *Y* 47 with 1/3 saturated KH_2PO_4 (20 ml.) and ninhydrin (500 mg.) for 15 min., removal of excess ninhydrin with alanine (225 mg.), refluxing for a further 15 min. and extraction of the pigments produced with $CHCl_3$ resulted in complete destruction of the active material.

(*c*) *Nitrous acid.* Treatment of *Y* 47 (0·8 ml.) with $NaNO_2$ (300 mg.) and 0·4N-HCl (10 ml.) overnight at room temperature resulted in complete loss of activity.

(*d*) *Osazone formation.* The filtrate after removal of phenylhydrazones from *Y* 15 (5 ml.) by treatment in the cold with phenylhydrazine hydrochloride (0·4 g.) and hydrated sodium acetate (2 g.) was boiled with glacial acetic acid (1 ml.) for 15 min. This treatment resulted in a partial inactivation.

Identification of the active material of Y 47 with a number of ninhydrin-positive components

Many of the techniques, examined before the above scheme for separation of the active material from yeast was perfected, showed defects which proved of interest in the further study of the extract. Attempts to remove the considerable amounts of sugar present by the use of 80% ethanol, baryta, methanol, pyridine, dialysed iron, calcium chloride and basic lead acetate were all discarded owing to a partition of activity between the two phases, and extraction of *Y* 47 with *p*-cresol had a similar effect.

These results might be explained as the simple partition of a single factor, or by the presence of more than one factor. Attempts were therefore made to fractionate *Y* 47 to find if the activity was associated with one or more of the components.

The complete destruction of activity by treatment with ninhydrin suggested that the active components were amino-acids or peptides. Two-dimensional partition chromatograms of *Y* 47 on paper, using phenol and collidine, showed the presence of twelve ninhydrin-positive compounds (see Fig. 1). Of these, five have been identified, by their relative positions and by the similarity of their individual positions, with aspartic acid, glutamic acid, serine, glycine and isoleucine. The remainder which could not be correlated with known amino-acids have been assigned the symbols, *S* 1, *S* 2, *S* 3, *P* 1, *P* 2, *P* 3 and *P* 4. The two spots *S* 2 and *S* 3 were very weak, apparently indicating a low concentration, while *P* 2 was present in high concentration.

Chromatograms obtained after hydrolysis in 6N-HCl for 18 hr. at 120° in a sealed tube showed that *P* 1, *P* 2, *P* 3 and *P* 4 are peptide in nature, since they completely disappeared from the chromatogram. Under these conditions the spots, *S* 1, *S* 2 and *S* 3 were stable. This implies that they are either free amino-acids or acid-stable peptides. Partial acid hydrolysis (6N-HCl for 18 hr. at 100°) caused the breakdown of *P* 2 and *P* 4, but *P* 1 and *P* 3 were not destroyed. Indeed, the concentration of *P* 1 and *P* 3, as indicated by the intensity of the chromatogram spots, increased, suggesting the possibility that they are simpler peptides derived from *P* 2 and *P* 4. Similar degrees of stability were observed after hydrolysis with 5% w/v ammonia for 18 hr. at 100° in a sealed tube. Separation of these various amino-acids and peptides was undertaken before an investigation of their nature was attempted.

Separation by ionophoresis. The technique of silica-gel ionophoresis devised by Consden, Gordon & Martin (1947) for the separation of amino-acids and short-chain peptides was applied to *Y* 47. The gel was made from sodium silicate, 30 ml. of a 4N solution being brought to pH 7·4 (bromothymol blue) with H_3PO_4, and the whole diluted to 400 ml. This was poured into the trough and allowed to set. The cathode perfusate was 0·125N-NaH_2PO_4 and the anode perfusate 0·34N-Na_3PO_4 in the ratio of 1/7; 5 ml. samples of *Y* 47 were mixed with appropriate quantities of sodium silicate solution to make the filling for the gutter. The potential applied was generally 120 V., occasionally 60 V., and the length of the gel about 50 cm.

The electrode perfusion liquids were circulated continuously (cf. Hall, 1948), and fractions collected as they passed over the anode gel/liquid interface. After runs varying in length from 5 to 22 hr. the material had separated into six distinct bands. Tables 4 and 5 give the activity, composition and mobility of these bands. Most of the activity was associated with two bands, *C* 2/1, which moved rapidly towards the anode, and *C* 2/4 + *C* 2/5 which contain material remaining in or near the gutter.

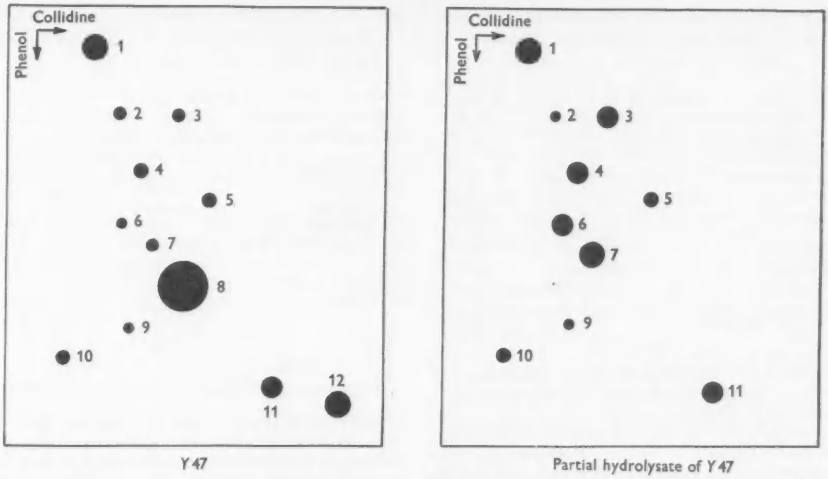


Fig. 1. Phenol/collidine chromatograms of *Y*47 and its partial hydrolysate after heating at 100° in 6N-HCl for 18 hr. The size of the circles indicates approximately the concentration of the ninhydrin-positive substances, which are respectively: (1) *S*1; (2) aspartic acid; (3) glutamic acid; (4) serine; (5) *P*3; (6) glycine; (7) *P*1; (8) *P*2; (9) *S*3; (10) *S*2; (11) isoleucine; (12) *P*4.

Table 4. *Separation of ninhydrin-positive components in Y*47 *by ionophoresis*

(Separation in a silica gel, pH 7·4; potential 120 V.)

Direction of movement	No. of component	Distance travelled in 4 hr. (mm.)	Breadth of band after 4 hr. (mm.)	Composition determined by partition chromatography
Towards anode	*C*2/1	70	24	*S*1
	*C*2/2	55	5	Glutamic acid, aspartic acid
	*C*2/3	35	6	Serine
	*C*2/4	15	14	*P*1, *P*2, *P*4, glycine and isoleucine
Towards cathode	*C*2/5	17	21	
	*C*2/6	31	6	*S*2

Table 5. *The activity of six components of Y*47 *separated by ionophoresis*

(*C* 2/4 and 5 also includes any material remaining in the gutter.)

Concentrations of additions, 10^{-x} ml./ml. of medium (x)	*Y*47	*C*2/1	*C*2/2	*C*2/3	*C*2/4 *C*2/5	*C*2/6
	(Activity numbers)					
1	100	0	0	0	67	0
2	100	1	0	0	100	17
3	100	67	17	33	67	0
4	100	67	33	17	67	1
5	100	33	33	1	33	1
6	100	17	17	1	17	0

A similar experiment with a strepogenin concentrate, kindly supplied by Dr D. W. Woolley (cf. Sprince & Woolley, 1945), showed no amino-acids even after prolonged application of the potential except within a range of a few millimetres of the gutter. This is in agreement with other observations that strepogenin, although containing two of the peptides present in *Y*47, does not contain any of the components moving more slowly in a phenol chromatogram, especially *S*1 (present in *C*2/1) and the free amino-acids glycine, serine, glutamic and aspartic acids.

Separation by partition chromatography. The technique devised by Dent (1947) for the separation of peptides from urine was used. Thirty-six spots of about 0·02 ml. each of *Y*47 were applied to a paper and chromatographed in phenol for 18 hr. The total length of the run was 342 mm. The two outside traces were removed and developed with ninhydrin and the main sheet cut accordingly into ten pieces. Of these, seven were initially judged to include all the ninhydrin-positive material present in *Y*47 while three, nos. 3, 5 and 9, represented the larger interspot spaces. The breadths of the bands and their composition as indicated by running subsequently in phenol or collidine are shown in Table 6.

Table 6. *Chromatographic characteristics of the various fractions of Y 47 run in phenol*

No.	Composition as judged by further chromatography	R_P of centre of band	Breadth of band (mm.)
1	*S*1, aspartic acid, glutamic acid, serine	0·06	42
2	Glutamic acid	0·15	19
3	—	—	15
4	Serine	0·24	29
5	—	—	15
6	Glycine, *P*1	0·40	37
7	Glycine	0·50	46
8	*P*2	0·62	36
9	—	—	30
10	*P*4, *S*2, isoleucine	0·92	83

Each fraction was separately extracted with water and tested. Most of the activity was observed in fractions nos. 1, 6 and 8, and slight activity in no. 10.

The refractionation of these fractions by chromatography either with phenol or collidine enabled pure samples of ten of the twelve ninhydrin-positive spots to be obtained, but two spots, *P*3 and *S*3, were so weak in the original chromatogram that they disappeared. Neither of these spots was associated with any detectable activity. The activity was confined to *S*1, *P*1, *P*2 and *S*2, the latter accounting for the slight activity of fraction 10, from the rest of which it could easily be separated by collidine chromatography.

Table 7 gives the activities of these fractions, none of which approaches that of the complete preparation; *S*1 is the most active of the components, while of the two peptides, *P*1 is by far the more active. All these fractions are required to produce maximum growth.

Table 7. *Activity of the unidentified fractions obtained from Y 47 by partition chromatography in phenol*

Concentration of additions, 10^{-x} ml./ml. of medium (x)	*Y*47	*S*1	*S*2	*P*1	*P*2	*P*3	*P*4	*S*1, *P*1, *P*2
	(Activity numbers)							
1	33	100	—	67	17	3	1	—
2	67	67	17	50	7	1	0	100
3	100	33	1	17	1	0	0	100
4	100	17	0	1	0	0	0	100
5	100	1	0	0	0	0	0	33

The effect of ophio L-α-*amino-acid oxidase.* This enzyme system (samples of which were kindly supplied by Dr E. A. Zeller of Basle), which is apparently without action upon peptides, was used to remove unnecessary amino-acids from *Y* 47 before it was appreciated that *S*1 was probably an amino-acid. Later experiments with the enzyme confirmed this fact. Treatment of the intact preparation with the oxidase appreciably lowered the activity and several of the spots observed in chromatograms disappeared. An acid-hydrolysed sample which retained partial activity owing to the presence of *S*1 lost all its remaining activity when treated with the enzyme. A typical experiment is recorded in Table 8.

Table 8. *The effect of ophio*-L-α-*amino-acid oxidase on the activity of Y 47 before and after hydrolysis in* 6N-HCl *at* 120° *for* 18 *hr.*

(1 ml. (0·2 mg.) enzyme in M/15 phosphate buffer, pH 7·4, was added to 0·1 ml. *Y* 47 or 0·1 ml. hydrolysate, and incubated at 37° for 8 hr.)

Concentration of additions, 10^{-x} ml./ml. of medium (x)	*Y*47	*Y*47 treated with oxidase	*Y*47 acid hydrolysate	*Y*47 hydrolysate treated with oxidase
	(Activity numbers)			
1	100	100	33	17
3	67	67	33	1
5	50	7	17	1
7	1	1	1	0

Attempted identification of some of the components

Of the active components in the preparation *Y* 47 only *P*2 was present in large enough concentration to permit the use of the multispot chromatographic technique. *S*1, slightly contaminated with serine, aspartic and glutamic acids, could be obtained by a large-scale method using a rectangular aluminium column, 18 in. long and $\frac{3}{4}$ in. square in cross-section, with a removable side. This was packed with paper pulp, saturated with water/phenol solution and, after the addition of up to 5 ml. of solution containing 1 g. of solid, the chromatogram was developed with phenol run slowly through from the top. When phenol containing ninhydrin-positive material appeared in the filtrate, the loose side was removed and a print of the position of the amino-acid bands taken on filter paper and developed with ninhydrin. The desired portions of the column were then excised and eluted with water or extracted in a Soxhlet apparatus. Separation of relatively large quantities of amino-acids into two fractions with high and low R_F values respectively was comparatively easy by this method, but the bands tended to be rather diffuse owing to non-specific flow through the interstices of the paper column (see Table 9). Adequate separation of the faster-running components was never practicable owing to this non-specific flow, but fractions rich in any one component could be obtained.

Table 9. *The separation of activity into two gross fractions by large-scale partition chromatography*

(Activity of both fractions together = 100 at all four dilutions.)

Concentration of additions, 10^{-x} ml./ml. medium (x)	Aqueous extract	Phenol extract
	(Activity numbers)	
1	67	67
2	67	67
3	7	67
4	0	33

*Examination of S*1. *S*1, running very slowly both in phenol and collidine (R_F's 0·09 and 0·15 respectively), occupies a position close to that of phosphorylserine (Dent,

1948) and phosphorylhydroxylysine (Gordon, 1948). Since serine is present in *Y* 47, and *S* 2, which has slight activity, gives a spot close to that of hydroxylysine, it seemed possible that *S* 1 might be one or other of the two phosphoryl derivatives. Estimation of the inorganic P content of *S* 1 before and after treatment with alkaline phosphatase showed that the material contained organic P, but no change occurred in the intensities of the spot for *S* 1, or in the activity of the preparation, nor did any more spots appear.

Examination of P 2. *P* 2, the only other component yet obtained pure in sufficient amount, was hydrolysed by 6N-HCl at 120° for 18 hr. and shown to consist of a polypeptide containing serine, glycine and glutamic acid, while *P* 1 was shown to be a partial hydrolysis product of this polypeptide.

Comparison of Y 47 *components with strepogenin*

Examination of the strepogenin concentrate by means of a one-dimensional phenol chromatogram showed that it contained only three ninhydrin-positive materials (owing to the small amount available no two-dimensional chromatograms were made). Two of the spots were close to *P* 1 and *P* 2, while the third ran slightly faster than the complex containing *P* 4, *S* 2, *S* 3 and isoleucine. Table 10 shows the activity of strepogenin compared with *Y* 47. Direct comparison with the figures given in Table 7 for the peptides concerned is not possible, since during extraction of the peptides from the chromatograms changes occur in the salt concentration, and dry weight determinations are of no significance.

Table 10. *Comparison of the activity of Y* 47 *and strepogenin in the growth of* Corynebacterium diphtheriae

Additions to medium, 10^{-x} mg./ml. (x)	Strepogenin	*Y* 47
	(Activity numbers)	
2	100	100
3	50	100
4	17	100
5	0·1	100

Preliminary examination of synthetic peptides

The following dipeptides showed considerable activity at the concentrations (μg./ml.) indicated: glutamylglutamic acid (0·01), glutamylglycine (0·03), glycylglutamic acid (0·1), leucylglutamic acid and glutamylalanine (from 5 to 10); alanylglutamic acid appeared to be inactive.

DISCUSSION

Several unidentified factors present in yeast extract or partial hydrolysates of protein have recently been described which appear to have some properties in common with the preparations described in this paper; the most notable being a factor required by certain strains of *Strep. lactis* (Smith, 1943), the 'sporogenes vitamin' of Peterson & Shull (1948), the factors required for maximum production of toxin by *Clostridium tetani* (Mueller & Miller, 1948) and strepogenin (Woolley, 1941). Peterson & Shull concluded from comparative assays of the two materials, using *Cl. sporogenes* and *Lb. casei* as test organisms, that their material and strepogenin were distinct. Since these two materials, and the yeast preparation described in this paper, contain a number of active components it is probable that they have one or more components in common; this is so for strepogenin and *Y* 47. The potency of a given preparation for a given organism may also depend upon the relative concentrations of the different components.

The preparation of a concentrate from yeast extract having considerable activity for the growth of a gravis strain of *C. diphtheriae*, type Dundee, has shown among other things the difference between this fraction and one, with similar physiological properties, obtained from liver. The active material from liver, after such mild purification as extraction with ethanol and ether, and the removal of some material on fuller's earth, was highly adsorbed on charcoal (norite) at pH 3. Crude yeast extracts, however, could be treated with norite without any significant loss in activity; and although some degree of adsorption from the final concentrate occurred, it was never complete. The concentrates, of which *Y* 47 is the most active so far obtained, are still mixtures of a variety of components, the activity being associated with two fractions (*P* 1 and *P* 2) which are probably peptide in nature and one (*S* 1) which is stable to acid hydrolysis. The failure to extract or adsorb only the active material from *Y* 47 compelled us to identify the components of the mixture and then separate these in turn.

Woolley (1946) has claimed that serylglycylglutamic acid has strepogenin activity of low order and that consequently it is either a portion of a more complex compound present in strepogenin concentrates, or is similar to, but not identical with, the active material. One of the peptides (*P* 2), common to both the strepogenin concentrate and *Y* 47, liberates serine, glycine and glutamic acid on hydrolysis. It is uncertain whether the amino-acids are joined in the order mentioned above, but the more stable peptide *P* 1, formed by partial hydrolysis of the tripeptide, occupies the position on a chromatogram assigned to serylglycine or glutamine by Dent (1947); the latter, however, can be excluded on the grounds of stability. It is, therefore, probable that serylglycylglutamic acid has some effect on *C. diphtheriae* metabolism, but until *P* 2 is obtained in larger amounts, and compared with a synthetic specimen of the tripeptide, it is impossible to be certain.

A number of synthetic glutamic acid peptides have been tested for activity, some of which induced

growth in our cultures of *C. diphtheriae*. The degree of activity was in no instance as great as that observed either with *Y* 47 or with the more active of its constituent factors. There is, of course, the possibility that other material having marked activity is present in these synthetic peptides at concentrations too low for chromatographic identification. The peptides *P* 1 and *P* 2, however, do not make up the whole of the activity for *C. diphtheriae*. *S* 1, the slow-moving stable material, is destroyed by the action of ophio-L-α-amino-acid oxidase and hence is presumably an amino-acid. On the other hand, since according to Zeller & Maritz (1944, 1945) the presence of OH and COOH groups in amino-acids prevents attack by the oxidase, the position of *S* 1 on chromatograms cannot be taken as indicating that it is a polycarboxylic or polyhydroxy amino-acid. Since this position is not altered after treatment with phosphatase, although inorganic phosphorus is produced, one must assume that the phosphate is liberated from some component giving no ninhydrin colour. The major part of the active material of *Y* 47 appears to be of a peptide or amino-acid nature, but there is some evidence for the existence of other active components as shown by the partial inactivation on osazone formation.

SUMMARY

1. The preparation is described of a concentrate from brewer's yeast which contains essential growth factors for certain strains of *Corynebacterium diphtheriae* gravis.

2. The activity of the whole preparation is associated with four of the twelve ninhydrin-positive components.

3. Most of the activity is associated with an amino-acid, *S* 1, and a peptide, *P* 1, while a second amino-acid, *S* 2, and another peptide, *P* 2, have slight activity.

4. The peptide *P* 2 consists of serine, glycine and glutamic acid.

5. A number of synthetic glutamic acid peptides also have activity as growth factors for *C. diphtheriae*.

We wish to acknowledge the co-operation of Dr M. McMillan, in the early stages of this work, and to thank Messrs Joshua Tetley and Son Ltd. for supplies of yeast, Messrs Roche Products Ltd. for gifts of material and the Medical Research Council for a grant during the course of the work; and also to acknowledge the gift of a number of synthetic peptides from Dr R. Consden of the Wool Industries Research Association.

REFERENCES

Chattaway, F. W., Happold, F. C. & Sandford, M. (1944). *Biochem. J.* **38**, 111.

Consden, R., Gordon, A. H. & Martin, A. J. P. (1947). *Biochem. J.* **41**, 590.

Dent, C. E. (1947). *Biochem. J.* **41**, 240.

Dent, C. E. (1948). *Biochem. J.* **43**, 169.

Dolby, D. E., Happold, F. C. & Sandford, M. (1944). *Nature, Lond.*, **153**, 619.

Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.

Evans, W. C., Handley, W. R. C. & Happold, F. C. (1939*a*). *Brit. J. exp. Path.* **20**, 41.

Evans, W. C., Handley, W. R. C. & Happold, F. C. (1939*b*). *Brit. J. exp. Path.* **20**, 396.

Gordon, A. H. (1948). *Nature, Lond.*, **162**, 778.

Hall, D. A. (1948). *Nature, Lond.*, **162**, 105.

Mueller, J. H. (1937*a*). *J. Bact.* **34**, 163.

Mueller, J. H. (1937*b*). *J. Bact.* **34**, 429.

Mueller, J. H. & Miller, P. A. (1948). *J. Bact.* **56**, 219.

Peterson, W. H. & Shull, G. M. (1948). *Arch. Biochem.* **18**, 69.

Smith, F. R. (1943). *J. Bact.* **46**, 369.

Sprince, A. & Woolley, D. W. (1945). *J. Amer. chem. Soc.* **67**, 1734.

Woolley, D. W. (1941). *J. exp. Med.* **73**, 487.

Woolley, D. W. (1946). *J. biol. Chem.* **166**, 783.

Zeller, E. A. & Maritz, A. (1944). *Helv. chim. Acta*, **27**, 1888.

Zeller, E. A. & Maritz, A. (1945). *Helv. chim. Acta*, **28**, 365.

A New Manometric Method for Determination of Respiratory Quotients

By H. LASER and Lord ROTHSCHILD

Molteno Institute and Department of Zoology, University of Cambridge

(*Received* 3 *January* 1949)

Manometric determinations of the respiratory quotient (R.Q.) require measurements of oxygen uptake, carbon dioxide production and, in the case of glycolysing cells, changes in the bicarbonate concentration of the medium. Several methods, due to Dickens & Simer (1930, 1931), Dixon & Keilin (1933), and Sumerson (1939), are available. Two identical, or practically identical, samples of the biological material are needed when these methods are used. The initial bicarbonate concentration is obtained by acidifying and killing one sample at the beginning of the experiment, while the change in bicarbonate concentration is determined by acidifying the other sample at the end of the experiment. Intermediate

values for the rate of oxygen uptake, carbon dioxide production and for the change in bicarbonate concentration during the experiment cannot be obtained. The R.Q. can therefore only be measured during one experimental period. Consequently, changes in R.Q. with time or with variations in the environmental conditions during the experiment cannot be observed. Such changes may be determined by using an adaptation of Warburg's indirect method (Laser & Rothschild, 1939), but this method is cumbersome when multiple determinations are involved.

A new apparatus with which changes in R.Q. can be measured on a single sample is described in this paper.

PRINCIPLE

The principle involves the sampling of known amounts of gas and liquid from a reaction vessel. One pair of samples is simultaneously removed from this vessel and connected with subsidiary manometers, in which their contents of gaseous CO_2 and bicarbonate are independently determined. After the initial determination at zero time (t_0), this procedure is repeated three times, the reaction in the main vessel continuing undisturbed. In this way changes in total CO_2 evolved and bicarbonate concentration of the medium are determined during three experimental periods. The corresponding changes occurring in the main vessel during the time between the taking of samples can be calculated from these data and from the readings of the manometer connected to the main vessel. As each of these readings registers the algebraic sum of the O_2 uptake and total CO_2 production, the oxygen consumption during each period can be calculated and the R.Q. determined. In the case of cell suspensions, during each experimental period the quantity of biological material in the main vessel is diminished by the amount taken out with each previous sample of the medium. This is taken into consideration in the equations which correlate the observed pressure changes with the amount of metabolizing material involved at each step. If tissue slices or whole organisms are used, the quantity of biological material remains unaltered throughout the experiment.

DESCRIPTION OF APPARATUS

Two versions of the apparatus (A and B) have been designed, differing in detail but employing the same principle.

Apparatus A. The reaction vessel is rectangular (Fig. 1, *A*). It is connected to a differential manometer which has a compensating vessel (Fig. 1, *B*) of about the same volume as the main vessel. The joint connecting the main vessel with its manometer is raised above the centre of the vessel, enabling the insertion of a turnable gas outlet (Fig. 1, *C*), and of a Keilin tube (Fig. 1, *D*), which is hung on a short length of platinum wire fused into the inner end of the gas outlet tube. The Keilin tube can be dislodged into the main vessel at any time during the experiment by rotating the gas outlet tube. The main vessel is provided at both ends with sampling devices, on which the principle of the method is based. Both ends are identical, and only one will be described in detail. It consists of a large tap, the sampling tap (Fig. 2, *C*), which is joined to the main vessel in such a way that its key (Fig. 2, *D*) forms the vertical side of the vessel. The key contains four invaginations, the sampling cups, which are in communication with the main vessel. In the apparatus shown in Fig. 2 the cups are in two rows vertically above each other, and at such a height that the lower ones (Fig. 2, c_0' and c_2') are below the level of the liquid. They are therefore completely filled with the fluid medium, while the upper ones (Fig. 2, c_0 and c_2) contain the gas in the main vessel. The key is hollow; its lower end and one side of the handle are cut open and therefore filled with water from the bath, to obtain satisfactory temperature equilibration and to eliminate buoyancy.

The sampling tap (Fig. 2, *C*) has an upper and a lower outlet tube (Fig. 2, *E* and *F*) at the levels of the sampling cups. These outlet tubes lead to joints (Fig. 3, *D*) through which each is connected with a subsidiary differential manometer having its own compensating vessel (Fig. 1, *G*). The complete apparatus therefore consists of five differential manometers, i.e. the main manometer and two subsidiary ones on each side. The **U**-tube of the main manometer is in the centre, while the two subsidiary manometers on each side are so arranged that one lies inside the other. Each sampling key is provided with indicator knobs (Fig. 2, *G*) corresponding to the positions of the sampling cups. By rotating the key outwards from the position in which all its cups communicate with the main vessel, one pair of sampling cups can be brought into communication with the corresponding outlet tubes (Fig. 4). Together with the outlet tube, each sampling cup then becomes the experimental vessel of a differential manometer, in one of which the CO_2 content of the gas sample is measured, and in the other, the bicarbonate content of the fluid.

Determination of the gaseous carbon dioxide. The upper outlet tube contains near its lower end a roll of filter paper (Fig. 2, *H*) soaked with KOH which absorbs all the CO_2 in the gas sample. The change of reading in the appropriate manometer determines the amount of CO_2 absorbed. A second roll of filter paper is inserted in the same outlet tube a little higher up (Fig. 2, *I*). This is wetted with water to adjust the water vapour tension in the subsidiary manometer to that of the main vessel. This prevents the development of pressure changes, not due to

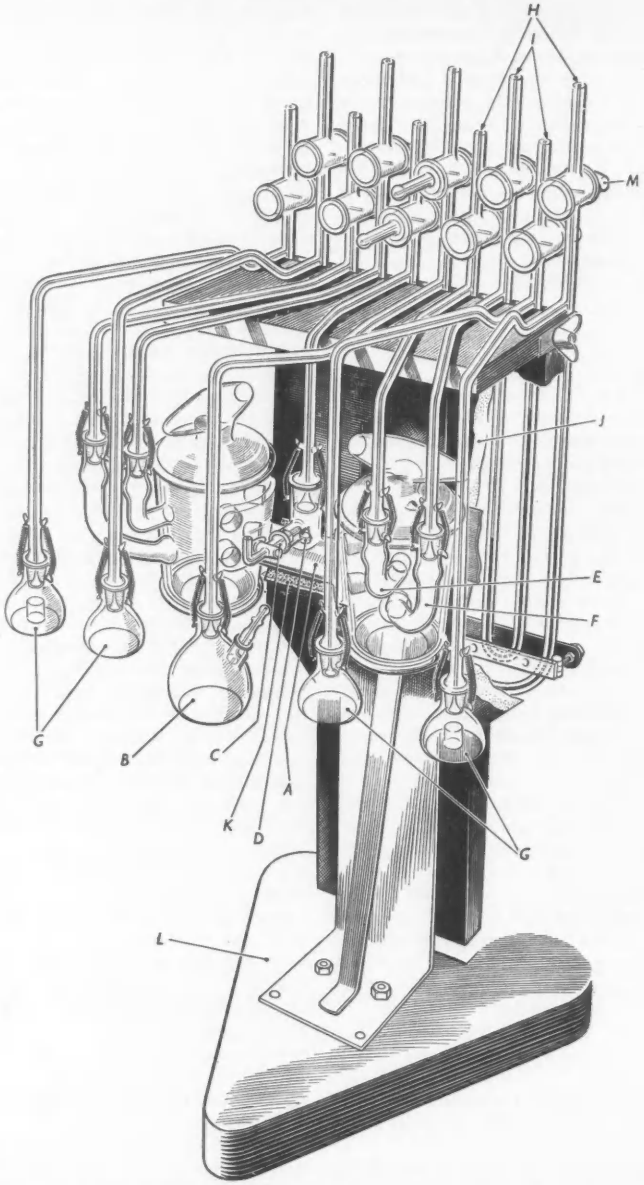


Fig. 1. Rear view of assembled apparatus (Type A). *A*, main reaction vessel; *B*, main compensating vessel; *C*, turnable gas outlet; *D*, Keilin tube; *E*, upper outlet tube (for CO_2 determination of gaseous sample); *F*, lower outlet tube (for bicarbonate-CO_2 determination of liquid sample); *G*, compensating vessels of subsidiary manometers; *H*, left subsidiary manometer (for determination of gaseous CO_2); *I*, left subsidiary manometer (for determination of bicarbonate CO_2); *J*, manometer board; *K*, rubber-lined clamp (partly cut away) for holding main vessel; *L*, stand; *M*, manometer tap. Length of centre manometer, base to tap, 275 mm.

CO_2, when the gas sample from the main vessel (which is saturated with water vapour) is connected with the manometer containing KOH.

Determination of bicarbonate content of the liquid sample. The lower outlet tube contains known amounts of mercury (Fig. 2, *J*) and an organic acid (Fig. 2, *K*). When the liquid-sampling cup is connected with this outlet tube, the mercury flows into the being superimposed on the existing readings in each manometer. From these readings the gaseous CO_2 and bicarbonate contents of the second pair of samples can be calculated. The changes in free CO_2 and bicarbonate concentration during the interval between taking the first and second samples can be calculated if the volumes of the sampling cups are known and, in the case of the gaseous samples, the

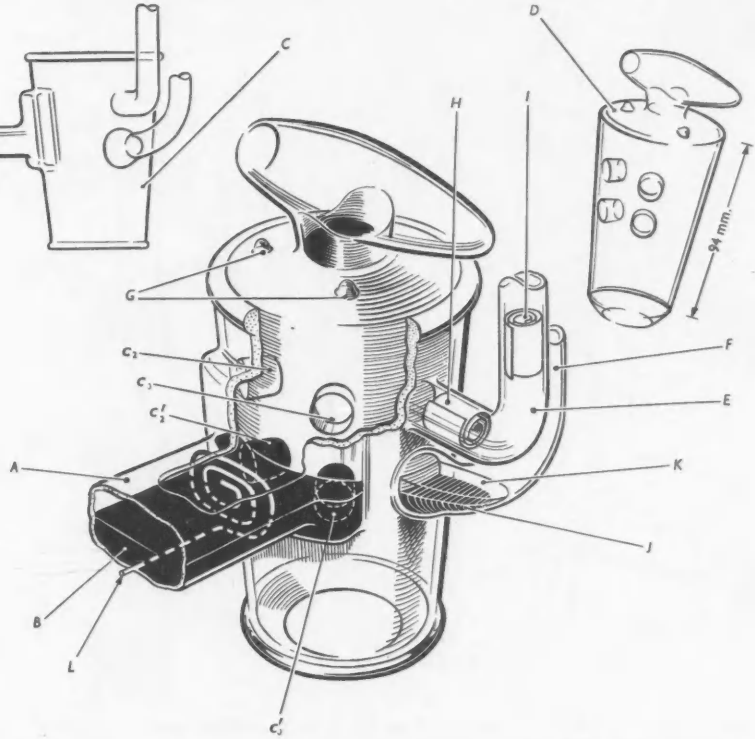


Fig. 2. Section of main vessel and sampling tap (Type A). *A*, Main reaction vessel; *B*, liquid (shown in black); *C*, barrel of sampling tap; *D*, key of sampling tap; *E*, upper outlet tube; *F*, lower outlet tube; *G*, indicator knobs; *H*, filter paper for KOH; *I*, filter paper for water; *J*, mercury; *K*, organic acid; *L*, platinum coil; c_0, c_0', c_2, c_2 sampling cups.

sampling cup and displaces some or all of its contents which are mixed with the acid. The amount of CO_2 expelled from the bicarbonate in the sample fluid can be calculated from the change in the reading of the appropriate manometer.

After an interval for CO_2 absorption and CO_2 expulsion the sampling key is turned a second time. The first pair of sampling cups are thereby removed from their manometers and the next pair connected with them. As neither the KOH in the upper outlet tube nor the acid in the lower one are exhausted, CO_2 absorption and expulsion are repeated, new pressures pressure in the main manometer at the time of sampling. As each sampling key contains two pairs of cups, four consecutive samples can be taken. In practice the sampling keys are turned alternately, the left one at times t_0 and t_2, and the right one at t_1 and t_3.

Apparatus B. This differs in one detail from apparatus A: the sampling cups are situated behind each other instead of next to each other. This is made possible by the introduction of two concentric keys in each sampling tap, the outer one forming a sleeve round the inner key. Fig. 3 shows the left

sampling tap. The inner key contains two invaginations, sampling cups c_0, c_0' and the outer keys two apertures constituting the sampling cups c_2, c_2'. At zero time the keys are in such a position that all sampling cups communicate with the main vessel. The lower invaginations (in the inner key) and the lower apertures (in the outer key) are then completely filled with liquid while the corresponding upper

APPLICATION

Technical application and sensitivity of the apparatus. The apparatus is not a microrespirometer and is therefore unsuitable for measuring small metabolic changes of the order of a few μl./hr. Such changes are too small to give significant differences in the samples. Changes of about 200–300 μl./hr. can be

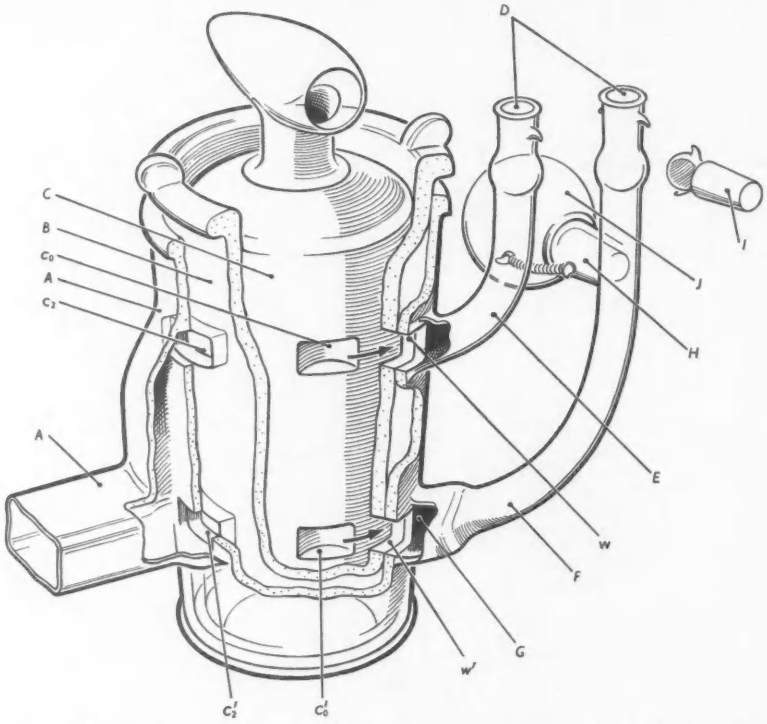


Fig. 3. Section of main vessel and sampling tap (Type B). *A*, main reaction vessel; *B*, outer key of sampling tap; *C*, inner key of sampling tap; *D*, joints of outlet tubes for insertion of subsidiary manometers; *E*, upper outlet tube; *F*, lower outlet tube; *G*, widened portion of lower outlet tube, to provide large surface for CO_2 expulsion from bicarbonate; *H*, joint in lower outlet tube for insertion of *I* or *J*; *I*, stopper (when worked with liquids of low bicarbonate content); *J*, extension (when worked with liquids of high bicarbonate content); c_0 and c_0', invaginations in inner key; c_2 and c_2', apertures in outer key; *w* and *w'*, apertures in outer key for connecting invaginations in inner key with the outlet tubes. Length of key, 95 mm.

invaginations and apertures contain the gas in the main vessel. The outer key also has two apertures opposite the lower and upper outlet tubes (Fig. 3, *w* and *w'*) through which the inner sampling cups communicate with the outlet tubes when the inner key is turned. By turning first the inner and later the outer key (Fig. 5), the inner and the outer sampling cups are successively connected with their corresponding outlet tubes. Here too the sampling is done alternately left and right.

measured at 15–30 min. intervals. These changes are large enough to be detected with accuracy in the samples, because the constants of the subsidiary manometers are small (about 0·5 for the CO_2-absorption manometer and 0·75 for the bicarbonate-CO_2 manometer with paraffin as the manometer fluid, and 0·65 and 1·0 with Brodie solution). The sensitivity can be increased by reducing the volume of the main vessel. The size of the sampling cups partly determines the sensitivity of the apparatus,

as the volume of the cup is directly proportional to the magnitude of the change which can be measured. With this in view, the gas-sampling cups were made to hold about 1·2–1·5 ml. and the liquid-sampling cups 0·9–1·0 ml. Comparison of the constants shows that the subsidiary manometers for the determination of the gaseous CO_2, while being sensitive enough to register small changes, are also large enough to hold the maximal pressures which can occur with 5 % CO_2 in the gas space. On the other hand, the bicarbonate-CO_2 manometers are not able to register more than about 200–300 μl. CO_2 according to the length of the manometer capillary. This is ample when liquids of low bicarbonate concentration, such as sea water, are used. For work with Ringer-

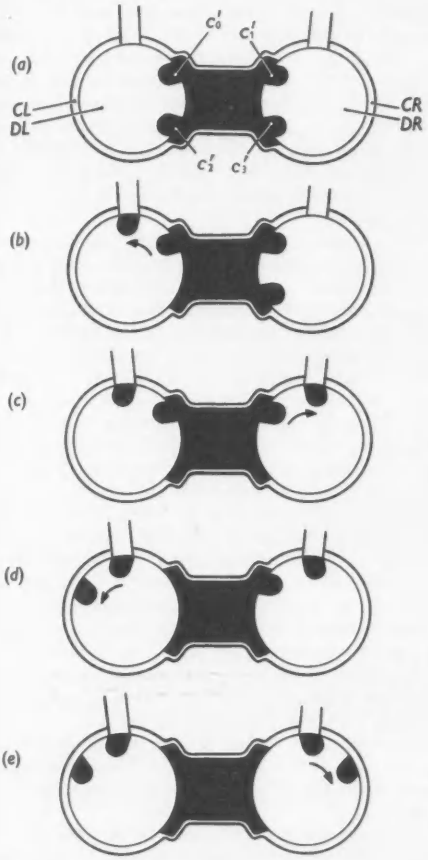


Fig. 4. Schematic cross section of main vessel (Type A) at the level of the liquid (black), illustrating the sampling procedure during the course of an experiment. *CL*, barrel of left sampling tap; *DL*, key of left sampling tap; *CR*, barrel of right sampling tap; *DR*, key of right sampling tap; c_0', and c_2' liquid sampling cups (left); c_1' and c_3', liquid sampling cups (right). The arrows inside the keys indicate the direction and sequence in which the keys are turned; (*a*) position during temperature equilibration; (*b*) position from t_0–t_1; (*c*) position from t_1–t_2; (*d*) position from t_2–t_3; (*e*) position from t_3–constant pressure in h_3. The same procedure applies automatically to the gaseous samples.

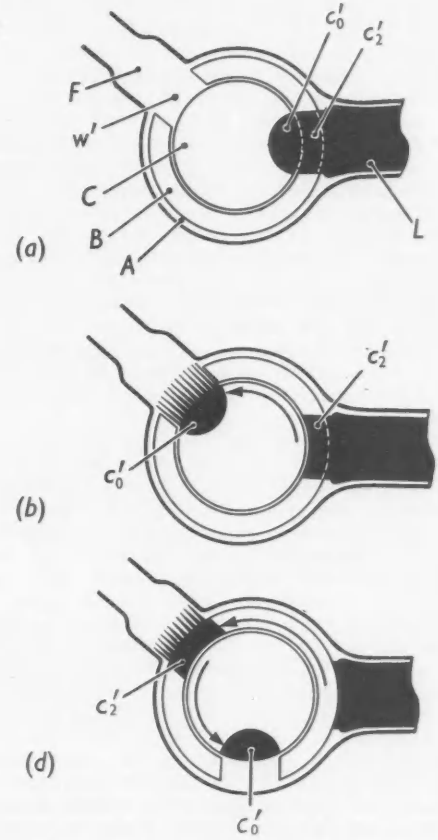


Fig. 5. Schematic cross section of left side of main vessel (Type B) illustrating the sampling procedure during the course of an experiment. *A*, barrel of sampling tap; *B*, outer key; *C*, inner key; *F*, lower outlet tube; *L*, liquid in main reaction vessel; c_0', liquid sampling cup in inner key; c_2', liquid sampling cup in outer key; *w'* aperture in outer key. The arrows inside the keys indicate the direction and sequence in which the keys are turned: (*a*) position during temperature equilibration; (*b*) position from t_0–t_1; (*d*) position from t_2–t_3. Positions (*c*) and (*e*) (see Fig. 4) illustrating sampling on the right side are omitted.

bicarbonate (about 550 μl. CO_2/ml.) and a liquid-sampling cup of 1 ml., the manometer would be too small. This difficulty can be overcome by decreasing the amount of liquid sampled (smaller cups in the key), or by increasing the manometer constant. There are limitations to decreasing the amount of sampled Ringer-bicarbonate solution, because it is not the absolute bicarbonate content but its change which has to be determined. The constant can be increased by using a manometer fluid with high density and/or by increasing the volume of the subsidiary manometer. For this purpose an extension can be fixed on the lower outlet tube (Fig. 3, *J*). The manometer fluid can also be levelled after the first bicarbonate-CO_2 determination. In that case the manometer only needs to register the pressure of one sample at a time. The minimum interval between sampling depends on the magnitude of metabolism and on the time taken for the expulsion of the bicarbonate CO_2 from the acidified liquid sample. Even with high bicarbonate concentrations, this was found to be complete in about 15 min. As the samples are taken from alternate sides, the effective interval can be reduced throughout the experiment to about 7·5 min./period. The interval between sampling at t_0 and t_1 can be still further reduced if the interval between t_1 and t_2 is correspondingly prolonged.

Biological application. The apparatus is applicable to the whole range of biological material which has so far been used in macromanometric work. In addition, it enables the study of many types of material for which manometric methods have been less suitable for the following reasons: (1) the absence of CO_2 in experiments with KOH interferes with their normal metabolism; (2) experiments in the presence of CO_2 using one of the existing methods are impossible, as it is not always easy to kill one sample at the beginning of the experiment; alternatively, two identical samples of the material cannot be obtained. This group includes plants, insects and their larvae, worms, small whole organs or muscle which could be stimulated *in situ*. The apparatus can also be adapted for the simultaneous determination of O_2 uptake, CO_2 production, and N_2 fixation by absorbing CO_2 in one and CO_2 and O_2 in the other outlet tube.

Metabolism without glycolysis. Many tissues and cells show no aerobic acid production. In such cases the four lower sampling cups are unnecessary. The apparatus can therefore be used without the subsidiary manometers for the determination of bicarbonate CO_2; or a simplified apparatus can be made having only two subsidiary manometers for the determination of the gaseous CO_2.

THEORY OF THE METHOD

Nomenclature. All symbols referring to the main manometer are expressed in capital letters and those referring to the subsidiary manometers in lower-case letters. They have the suffixes 0, 1, 2 and 3. Some of the constants have the additional suffixes O_2 or CO_2. In the case of the main manometer the suffixes refer to the time at which the readings are taken. The amounts of gas (V_G) and of fluid (V_F) in the main vessel and the constants change at each time of sampling. If V represents the total volume of the main vessel including the eight sampling cups, and if V_{G_0} and V_{F_0}, V_{G_0} and V_{F_1}, etc. represent the gas and fluid volumes at t_0, t_1, etc., then H_2 is the reading on the main manometer at t_2 and $K_{2:O_2}$ the O_2 constant applicable for the second time interval (t_1–t_2) based on V_{G_2} and V_{F_2}.

The gas-sampling cups are called c_0 and c_2 on the left side and on the right side c_1 and c_3, the appropriate manometer readings being h_0 and h_2 (left) and h_1 and h_3 (right). The corresponding liquid-sampling cups and readings are indexed with a superscript $'$. Thus the liquid-sampling cups are called c_0' and c_2' (left), c_1' and c_3' (right) and the readings h_0' and h_2' (left) and h_1' and h_3' (right). The volumes and constants of the subsidiary manometers also change at each time of sampling according to the volume of the sampling cup with which they become connected. As all the gaseous CO_2 which is sampled and measured in the subsidiary manometers is absorbed by KOH, the constants applicable in this case are those converting the pressure produced by a free gas and do not include the solubility factor α_{CO_2}. They are called k_0, k_1, k_2 and k_3. The constants used for the determination of bicarbonate CO_2 contain the factor α_{CO_2} and are called $k'_{0:CO_2}$, $k'_{1:CO_2}$, $k'_{2:CO_2}$ and $k'_{3:CO_2}$. The suffixes in the subsidiary manometers refer to the time of sampling. Thus h_2' and $k'_{2:CO_2}$ refer to the reading and the CO_2 constant of the subsidary manometer with respect to the liquid removed at t_2, but read at a convenient time during the experiment when the CO_2 expulsion from bicarbonate is complete.

In determining the gaseous CO_2 only the free CO_2 in the main vessel at the time of sampling is measured. This does not include the CO_2 which has gone into solution in the fluid in the main vessel. The free CO_2 has therefore to be converted into total CO_2 by multiplication at each step with the appropriate factor $\frac{K_{1:CO_2}}{K_1}$ or $\frac{K_{2:CO_2}}{K_2}$ or $\frac{K_{3:CO_2}}{K_3}$, where $K_{1:CO_2}$, $K_{2:CO_2}$ and $K_{3:CO_2}$ are the CO_2 constants and K_1, K_2 and K_3 are the corresponding constants from which the factor $V_F\,\alpha_{CO_2}$ has been omitted. Table 1 then applies to the readings, volumes and constants of the apparatus.

Determination of gaseous carbon dioxide. Suppose that the gas in the main vessel contains no CO_2 and that at t_0 the main manometer shows no pressure difference. Connecting the sampling cup c_0 with its subsidiary manometer will then produce no pressure

Table 1. *Relations between constants of the apparatus*

A. Main manometer

Time (t)	Reading	Change in reading	Gas volume	Liquid volume	Constants K	K_{O_2}	K_{CO_2}
0	H_0	—	V_{G_0}	V_{F_0}	K_0	—	—
1	H_1	$H_1-H_0=\Delta H_1$	$V_{G_0}-c_0=V_{G_1}$	$V_{F_0}-c_0'=V_{F_1}$	K_1	$K_{1:O_2}$	$K_{1:CO_2}$
2	H_2	$H_2-H_1=\Delta H_2$	$V_{G_0}-(c_0+c_1)=V_{G_2}$	$V_{F_0}-(c_0'+c_1')=V_{F_2}$	K_2	$K_{2:O_2}$	$K_{2:CO_2}$
3	H_3	$H_3-H_2=\Delta H_3$	$V_{G_0}-(c_0+c_1+c_2)=V_{G_3}$	$V_{F_0}-(c_0'+c_1'+c_2')=V_{F_3}$	K_3	$K_{3:O_2}$	$K_{3:CO_2}$

B. Subsidiary manometer

Sampled time (t)	Subsidiary manometer	Outlet tube	Sampling cup	Manometer reading	Constant
0	1	t_{ul}*	c_0	h_0	k_0
0	2	t_{ll}	c_0'	h_0'	$k'_{0:CO_2}$
1	3	t_{ur}†	c_1	h_1	k_1
1	4	t_{lr}	c_1'	h_1'	$k'_{1:CO_2}$
2	1	t_{ul}	c_2	h_2	k_2
2	2	t_{ll}	c_2'	h_2'	$k'_{2:CO_2}$
3	3	t_{ur}	c_3	h_3	k_3
3	4	t_{lr}	c_3'	h_3'	$k'_{3:CO_2}$

t = outlet tube (Figs. 2 and 3, *E* and *F*); ul = upper left; ur = upper right; ll = lower left; lr = lower right.

* In apparatus *B* this becomes $t_{ul}+w_l$. † In apparatus *B* this becomes $t_{ur}+w_r$.

change in that manometer. On the other hand, if at t_0 a pressure exists in the main manometer, the introduction of the gas volume contained in c_0 into the subsidiary manometer will produce a pressure difference depending on the pressure at which the gas is introduced and on the constant of the subsidiary manometer. The change of reading in the subsidiary manometer will be

$$\Delta_0 = H_0 \frac{K_0 c_0}{V_{G_0} k_0}.$$

Similarly, on sampling gas at t_1, t_2 and t_3, the following changes of pressure in the subsidiary manometers will be produced, depending on the pressure in the main vessel at these times:

$$\Delta_j = H_j \frac{K_j c_j}{V_{G_j} k_j} \quad (j = 1, 2 \text{ or } 3).$$

Theoretically the Δ equations need two corrections to account for the solubility of gases other than CO_2 in the liquid contained in the subsidiary manometer, and the changes in this factor with variations in the relative amounts of these gases due to the O_2 uptake during the experiment. Both correction factors are small as the amount of liquid in the subsidiary manometer is small (0·4 ml.) and the gases concerned, mainly O_2, N_2, CO and H_2, have a low solubility. k_0 differs from $k_{0:O_2}$, $k_{0:N_2}$, etc. only by 0·001. Apart from the magnitude of this factor, it can safely be omitted as the CO_2 changes are calculated as differences and are unaffected by the omission of the same correction factor in all four determinations. However, because of the variations in the ratios of these gases during the experiment, this factor alters very slightly during the experiment. The Δ-values calculated from the above equations differ by less than 0·1 mm. from the theoretical figure for an O_2 uptake of 500 μl. in the main vessel. Both factors can therefore be omitted without affecting the accuracy of the result.

If the sampled gas contains CO_2, the Δ-values have to be subtracted from the corresponding readings to evaluate the amount of CO_2 present. If $x_{0:CO_2}$ is the amount of CO_2 present in c_0,

$$x_{0:CO_2} = (h_0 - \Delta_0)\, k_0,$$

or

$$x_{0:CO_2} = h_0 k_0 - H_0 \frac{K_0 c_0}{V_{G_0}}.$$

In this equation the term k_0, contained in Δ_0, cancels out. Similarly, the terms k_1, k_2 and k_3 contained in Δ_1, Δ_2 and Δ_3, cancel out in the subsequent equations for $x_{1:CO_2}$, $x_{2:CO_2}$ and $x_{3:CO_2}$ which will contain the factors

$$H_j = \frac{K_j c_j}{V_{G_j}} \quad (j = 1, 2 \text{ or } 3).$$

Thus

$$x_{1:CO_2} = h_1 k_1 - H_1 \frac{K_1 c_1}{V_{G_1}}.$$

The absorption of CO_2 by KOH produces a negative reading in the subsidiary manometer. To conform with the usual sign conventions the equations for $x_{0:CO_2}$ and $x_{1:CO_2}$ are converted into

$$x_{0:CO_2} = H_0 \frac{K_0 c_0}{V_{G_0}} - h_0 k_0, \tag{1}$$

$$x_{1:CO_2} = H_1 \frac{K_1 c_1}{V_{G_1}} - h_1 k_1. \tag{2}$$

$x_{1:CO_2}$ represents the total amount of CO_2 in c_1 which includes the amount of CO_2 present in c_1 at t_0. Therefore the change of CO_2 in c_1 between t_0 and t_1 is

$$\Delta x_{1:CO_2} = x_{1:CO_2} - x_{0:CO_2} \frac{c_1}{c_0}. \tag{3}$$

On connecting c_2 with its subsidiary manometer a new pressure is superimposed on the existing reading h_0. This does not remain as an unaltered fraction of the new reading h_2, because part of the system in which the pressure h_0 has previously been produced, i.e. c_0, has been removed. The fraction of h_0 included in h_2 is*

$$\frac{h_0 k_0}{k_2}\frac{t_{ul}}{t_{ul}+c_0}.$$

Similarly, on determining $x_{3:CO_2}$, h_3 will contain a fraction of the previous reading h_1, this fraction being†

$$\frac{h_1 k_1}{k_3}\frac{t_{ur}}{t_{ur}+c_1}.$$

These factors have to be subtracted from h_2 and h_3 for the evaluation of $x_{2:CO_2}$ and $x_{3:CO_2}$:

$$x_{2:CO_2}=H_2\frac{K_2 c_2}{V_{G_2}}-\left[h_2-\frac{h_0 k_0}{k_2}\frac{t_{ul}}{t_{ul}+c_0}\right]k_2$$

or
$$x_{2:CO_2}=H_2\frac{K_2 c_2}{V_{G_2}}+h_0\frac{k_0 t_{ul}}{t_{ul}+c_0}-h_2 k_2, \qquad (4)$$

$$\Delta x_{2:CO_2}=x_{2:CO_2}-x_{1:CO_2}\frac{c_2}{c_1}, \qquad (5)$$

$$x_{3:CO_2}=H_3\frac{K_3 c_3}{V_{G_3}}+h_1\frac{k_1 t_{ur}}{t_{ur}+c_1}-h_3 k_3, \qquad (6)$$

$$\Delta x_{3:CO_2}=x_{3:CO_2}-x_{2:CO_2}\frac{c_3}{c_2}. \qquad (7)$$

The Δ-values for CO_2 so far obtained, (3), (5), (7), are those occurring in c_1 from t_0 to t_1, in c_2 from t_1 to t_2, and in c_3 from t_2 to t_3. These have to be converted into the corresponding changes/ml. of suspension, if cell suspensions are used (the amount of which changes with each time of sampling) or changes/mg. of weight or dry weight, if material such as tissue slices or whole organisms are used (the amount of which remains constant throughout the experiment). The latter equations are suffixed a. Thus:

ΔX_{CO_2}/ml. cell suspension

$$\Delta X_{j:CO_2}=\Delta x_{j:CO_2}\frac{V_{G_j}}{V_{F_j}}\frac{K_{j:CO_2}}{c_j K_j} \quad (j=1,\ 2 \text{ or } 3),$$

(8), (9), (10)

ΔX_{CO_2}/unit weight

$$\Delta X_{j:CO_2}=\frac{\Delta x_{j:CO_2}}{\text{wt.}}\frac{V_{G_j}}{c_j}\frac{K_{j:CO_2}}{K_j} \quad (j=1,\ 2 \text{ or } 3).$$

(8a), (9a), (10a)

* In apparatus B this expression becomes

$$\frac{h_0 k_0}{k_2}\frac{t_{ul}}{t_{ul}+w_l+c_0}.$$

† In apparatus B this expression becomes

$$\frac{h_1 k_1}{k_2}\frac{t_{ur}}{t_{ur}+w_r+c_1}.$$

Determination of bicarbonate carbon dioxide. Equations 11–14 express the bicarbonate concentrations as μl. CO_2/ml. fluid. They are calculated for the case in which the amount of mercury introduced into the subsidiary manometers on each side (Fig. 2, J) is greater than the volume of fluid with which the mercury changes place on sampling at t_0 and at t_1. In this case the total volume of fluid in c_0' and c_1' is forced up into the lower outlet tubes of the sampling taps and remains there during the determination for the samples from c_2' and c_3'. This facilitates the calculation of $x_{2:B}$ and $x_{3:B}$:

$$x_{0:B}=\frac{h_0' k'_{0:CO_2}}{c_0'}, \qquad (11)$$

$$x_{1:B}=\frac{h_1' k'_{1:CO_2}}{c_1'}, \qquad (12)$$

$$x_{2:B}=\frac{h_2' k'_{2:CO_2}-h_0' k'_{0:CO_2}}{c_2'}, \qquad (13)$$

$$x_{3:B}=\frac{h_3' k'_{3:CO_2}-h_1' k'_{1:CO_2}}{c_3'}. \qquad (14)$$

The change in bicarbonate concentration corresponds to acid production or glycolysis (X_G):

ΔX_G/ml. cell suspension

$$\Delta X_{1:G}=x_{0:B}-x_{1:B}, \qquad (15)$$

$$\Delta X_{2:G}=x_{1:B}-x_{2:B}, \qquad (16)$$

$$\Delta X_{3:G}=x_{2:B}-x_{3:B}; \qquad (17)$$

ΔX_G/unit weight

$$\Delta X_{1:G}=\frac{V_{F_1}}{\text{wt.}}(x_{0:B}-x_{1:B}), \qquad (15a)$$

$$\Delta X_{2:G}=\frac{V_{F_2}}{\text{wt.}}(x_{1:B}-x_{2:B}), \qquad (16a)$$

$$\Delta X_{3:G}=\frac{V_{F_3}}{\text{wt.}}(x_{2:B}-x_{3:B}). \qquad (17a)$$

Respiratory carbon dioxide. The respiratory CO_2 (resp. CO_2) is obtained by deducting the glycolytic CO_2 from the total CO_2 for each period; thus $\Delta X^{\text{resp.}}_{j:CO_2}$/ml. cell suspension or per unit weight:

$$\Delta X^{\text{resp.}}_{j:CO_2}=\Delta X_{j:CO_2}-X_{j:G} \quad (j=1,\ 2 \text{ or } 3).$$

(18), (19), (20), (18a), (19a), (20a).

Determination of oxygen uptake. The O_2 uptake is calculated from the readings of the main manometer. The following equations apply:

ΔX_{O_2}/ml. cell suspension

$$\frac{\Delta H_1}{V_{F_1}}=\frac{\Delta X_{1:O_2}}{K_{1:O_2}}+\frac{\Delta X_{1:CO_2}}{K_{1:CO_2}},$$

whence

$$\Delta X_{j:O_2}=\left[\frac{\Delta H_j}{V_{F_j}}-\frac{\Delta X_{j:CO_2}}{K_{j:CO_2}}\right]K_{j:O_2} \quad (j=1,\ 2 \text{ or } 3).$$

(21), (22), (23)

ΔX_{O_2}/unit weight

$$\frac{\Delta H_1}{\text{wt.}} = \frac{\Delta X_{1:O_2}}{K_{1:O_2}} + \frac{\Delta X_{1:CO_2}}{K_{1:CO_2}},$$

whence

$$\Delta X_{f:O_2} = \left[\frac{\Delta H_f}{\text{wt.}} - \frac{\Delta X_{f:CO_2}}{K_{f:CO_2}}\right] K_{f:O_2} \quad (j = 1, 2 \text{ or } 3). \qquad (21a), (22a), (23a)$$

The respiratory quotient is given by the ratios in Table 2.

Table 2. *Calculation of respiratory quotients*

(The numbers refer to equation numbers.)

	R.Q. with	
t	Cell suspensions	Tissue slices, or whole organism
0–1	18/21	18a/21a
1–2	19/22	19a/22a
2–3	20/23	20a/23a

Table 3. *Symbols to be introduced into equations* 1–23

Symbol	Equation	Introduced for	Symbol	Equation	Introduced for
A	1	$\frac{K_0 c_0}{V_{G_0}}$	O	11	$\frac{k'_{0:CO_2}}{c_0'}$
B	2	$\frac{K_1 c_1}{V_{G_1}}$	P	12	$\frac{k'_{1:CO_2}}{c_1'}$
C	4	$\frac{K_2 c_2}{V_{G_2}}$	Q	13	$\frac{k'_{2:CO_2}}{c_2'}$
D	6	$\frac{K_3 c_3}{V_{G_3}}$	R	13	$\frac{k'_{0:CO_2}}{c_3'}$
E_l	4	$\frac{k_0 t_{ul}}{t_{ul}+c_0}$	S	14	$\frac{k'_{3:CO_2}}{c_3'}$
E_r	6	$\frac{k_1 t_{ur}}{t_{ur}+c_1}$	T	14	$\frac{k'_{1:CO_2}}{c_3'}$
F	3	$\frac{c_1}{c_0}$	U	21 21a	$\frac{K_{1:O_2}}{V_{F_1}}$
G	5	$\frac{c_2}{c_1}$	V	21	$\frac{K_{1:O_2}}{K_{1:CO_2}}$
H	7	$\frac{c_3}{c_2}$	W	22	$\frac{K_{2:O_2}}{V_{F_2}}$
I	8	$\frac{V_{G_1} K_{1:CO_2}}{V_{F_1} c_1 K_1}$	X	22 22a	$\frac{K_{2:O_2}}{K_{2:CO_2}}$
J	9	$\frac{V_{G_2} K_{2:CO_2}}{V_{F_2} c_2 K_2}$	Y	23	$\frac{K_{3:O_2}}{V_{F_3}}$
K	10	$\frac{V_{G_3} K_{3:CO_2}}{V_{F_3} c_3 K_3}$	Z	23 23a	$\frac{K_{3:O_2}}{K_{3:CO_2}}$
L	8a	$\frac{V_{G_1} K_{1:CO_2}}{c_1 K_1}$			
M	9a	$\frac{V_{G_2} K_{2:CO_2}}{c_2 K_2}$			
N	10a	$\frac{V_{G_3} K_{3:CO_2}}{c_3 K_3}$			

Simplification of calculations. The equations contain a number of factors which are constant for a given set of experimental conditions at a given temperature. These factors can be calculated and expressed by symbols (Table 2), the introduction of which facilitates subsequent calculation. The equations can then be written down in the following way, those suffixed a referring to unit weight:

$$x_{0:CO_2} = \mathbf{A}H_0 - h_0 k_0, \qquad \text{(I)}$$

$$x_{1:CO_2} = \mathbf{B}H_1 - h_1 k_1, \qquad \text{(II)}$$

$$\Delta x_{1:CO_2} = \text{(II)} - \mathbf{F}\ \text{(I)}, \qquad \text{(III)}$$

$$x_{2:CO_2} = \mathbf{C}H_2 + \mathbf{E}_l h_0 - h_2 k_2, \qquad \text{(IV)}$$

$$\Delta x_{2:CO_2} = \text{(IV)} - \mathbf{G}\ \text{(II)}, \qquad \text{(V)}$$

$$x_{3:CO_2} = \mathbf{D}H_3 + \mathbf{E}_r h_1 - h_3 k_3, \qquad \text{(VI)}$$

$$\Delta x_{3:CO_2} = \text{(VI)} - \mathbf{H}\ \text{(IV)}; \qquad \text{(VII)}$$

$$\Delta X_{1:CO_2} = \mathbf{I}\ \text{(III)}, \qquad \text{(VIII)}$$

$$\Delta X_{2:CO_2} = \mathbf{J}\ \text{(V)}, \qquad \text{(IX)}$$

$$\Delta X_{3:CO_2} = \mathbf{K}\ \text{(VII)}, \qquad \text{(X)}$$

$$\Delta X_{1:CO_2} = \frac{\mathbf{L}\ \text{(III)}}{\text{wt.}}, \qquad \text{(VIII}a\text{)}$$

$$\Delta X_{2:CO_2} = \frac{\mathbf{M}\ \text{(V)}}{\text{wt.}}, \qquad \text{(IX}a\text{)}$$

$$\Delta X_{3:CO_2} = \frac{\mathbf{N}\ \text{(VII)}}{\text{wt.}}; \qquad \text{(X}a\text{)}$$

$$x_{0:B} = \mathbf{O}h'_0, \qquad \text{(XI)}$$

$$x_{1:B} = \mathbf{P}h'_1, \qquad \text{(XII)}$$

$$x_{2:B} = \mathbf{Q}h'_2,\ -\mathbf{R}h'_0, \qquad \text{(XIII)}$$

$$x_{1:B} = \mathbf{S}h'_3 - \mathbf{T}h'_1; \qquad \text{(XIV)}$$

$$\Delta X_{1:G} = \text{(XI)}\text{–}\text{(XII)}, \qquad \text{(XV)}$$

$$\Delta X_{2:G} = \text{(XII)}\text{–}\text{(XIII)}, \qquad \text{(XVI)}$$

$$\Delta X_{3:G} = \text{(XIII)}\text{–}\text{(XIV)}, \qquad \text{(XVII)}$$

$$\Delta X_{1:G} = \frac{V_{F_1}}{\text{wt.}}\ \text{(XI–XII)}, \qquad \text{(XV}a\text{)}$$

$$\Delta X_{2:G} = \frac{V_{F_2}}{\text{wt.}}\ \text{(XII–XIII)}, \qquad \text{(XVI}a\text{)}$$

$$\Delta X_{3:G} = \frac{V_{F_3}}{\text{wt.}}\ \text{(XIII–XIV)}; \qquad \text{(XVII}a\text{)}$$

$$\Delta X^{\text{resp.}}_{1:CO_2} = \text{(VIII)}\text{–}\text{(XV)}, \qquad \text{(XVIII)}$$

$$\Delta X^{\text{resp.}}_{2:CO_2} = \text{(IX)}\text{–}\text{(XVI)}, \qquad \text{(XIX)}$$

$$\Delta X^{\text{resp.}}_{3:CO_2} = \text{(X)}\text{–}\text{(XVII)}, \qquad \text{(XX)}$$

$$\Delta X^{\text{resp.}}_{1:CO_2} = \text{(VIII}a\text{)}\text{–}\text{(XV}a\text{)}, \qquad \text{(XVIII}a\text{)}$$

$$\Delta X^{\text{resp.}}_{2:CO_2} = \text{(IX}a\text{)}\text{–}\text{(XVI}a\text{)}, \qquad \text{(XIX}a\text{)}$$

$$\Delta X^{\text{resp.}}_{3:CO_2} = \text{(X}a\text{)}\text{–}\text{(XVII}a\text{)}; \qquad \text{(XX}a\text{)}$$

$$\Delta X_{1:O_2} = U\Delta H_1 - V \text{ (VIII)}, \qquad \text{(XXI)}$$

$$\Delta X_{2:O_2} = W\Delta H_2 - X \text{ (IX)}, \qquad \text{(XXII)}$$

$$\Delta X_{3:O_2} = Y\Delta H_3 - Z \text{ (X)}, \qquad \text{(XXIII)}$$

$$\Delta X_{1:O_2} = \frac{\Delta H_1 K_{1:O_2}}{\text{wt.}} - V \text{ (VIII}a\text{)}, \qquad \text{(XXI}a\text{)}$$

$$\Delta X_{2:O_2} = \frac{\Delta H_2 K_{2:O_2}}{\text{wt.}} - X \text{ (IX}a\text{)}, \qquad \text{(XXII}a\text{)}$$

$$\Delta X_{3:O_2} = \frac{H_3 K_{3:O_2}}{\text{wt.}} - Z \text{ (X}a\text{)}. \qquad \text{(XXIII}a\text{)}$$

Equations I–VII and XI–XIV apply equally to cell suspensions and tissue slices or organisms. Equations VIII–XXIII and VIII*a*–XXIII*a* express the values for total CO_2 output, glycolytic CO_2, respiratory CO_2 and O_2 uptake as μl./ml. cell suspension or μl./unit weight for each separate period of sampling. In the case of non-glycolysing tissues, the formulae for bicarbonate-CO_2 (XI–XIV), glycolysis (XV–XVII) and for the determination of the respiratory CO_2 (XVIII–XX) do not apply. Equations VIII–X give directly the respiratory CO_2, and only equations I–X and XXI–XXIII need be calculated. Once the symbols have been calculated, the evaluation of results is obtained from equations I–XXIII. If this procedure is followed, calculation of an entire experiment may be completed in about 30 min.

MANIPULATION OF THE APPARATUS. RESULTS

The assembly and manipulation of the apparatus are simple. The wide manometer board (Fig. 1, *J*) has at its back two slides which are arranged to fit into two neighbouring brackets on the shaker bar of the tank. The manometers are not fixed to the board, but are kept in a wooden stand which is provided with slots to fit the U-shaped parts of the manometers. The main vessel is kept in a wooden block. When assembling the apparatus the vessel is not put on to the manometers as is usual, but the manometers, with the compensating vessels in position, are slid into their joints after the vessel has been fixed in a rubber-lined clamp (Fig. 1, *K*) at the back of the board. An alternative type of clamp secures the vessel round the sampling taps to allow illumination of the vessel. All compensating vessels contain water, those connected to the subsidiary manometers for the determination of gaseous CO_2 having in addition KOH on filter papers in their centre wells, to obtain the same water vapour tension as in the experimental side of the manometer. The sampling keys are greased and so inserted into the taps that all sampling cups are in open communication with the main vessel. The lower rolls of filter paper are then inserted into the upper outlet tubes and soaked with 0·2 ml. 10% KOH. The upper rolls of filter paper are next inserted and wetted with 0·2 ml. water. (A standard size of Whatman no. 2 filter paper is being used, 15 × 75 mm., the volume of which is determined and taken into account when calculating the constants.) Redistilled mercury (3 ml.)—care must be taken that no air is trapped—and tartaric acid (0·5 ml. of 0·5 N) are run into each lower outlet tube. The main vessel is then filled with the amount of suspension or fluid necessary to fill and completely cover the lower sampling cups. Tissue slices or small organisms must be prevented from lodging in the lower sampling cups. This is done by placing a platinum coil in the vessel (schematically drawn in Fig. 2, *L*) or by putting the material in a small platinum-wire basket. Ringer-bicarbonate solution should be equilibrated with 5% (v/v) CO_2/O_2 before putting it into the vessel. The gas-outlet tap is introduced, and, if necessary, the Keilin tube attached. The apparatus is assembled on a solid stand (Fig. 1, *L*) which has two brackets fitting the slides of the manometer board. The vessel is secured in the clamp at the back of the manometer board and the central manometer inserted. The greased joint is worked in by gentle movement of the manometer and fixed with springs. If gassing is required or continuation of gassing with a CO_2-gas mixture, this is done in the ordinary way at this stage. Next, the outer and then the inner subsidiary manometers are slid into position in their joints and fixed. All manometers are secured in front of the manometer board by two rubber-lined clamps across the width of the board. The gassing is stopped, the gas-outlet tap closed and the manometer transferred into the bath. If necessary, gassing can be done or continued while the manometer is shaken in the bath. In this case the gas outlet (Fig. 1, *C*) is prolonged above the surface of the water by a length of rubber tubing. It is advisable to gas both sides of the main manometer in order to prevent excessive movement of the manometer fluid, which, with the narrow capillary (area 0·25 sq.mm.), may affect later readings because of the slow draining of traces of manometer fluid. Temperature equilibration is proceeded with as in any differential manometer. The subsidiary manometers do not need gassing. It is, however, important to shut all manometers simultaneously with the compensating vessel of the main vessel. This is done when the position of the fluid in the main manometer is adjusted before the reading at t_0. In this way small temperature differences during the experimental period do not affect the later readings of the samples.

The four subsidiary manometers should show no pressure differences up to the moment when they are connected with one of the sampling cups. The position of the sampling cups during the course of the experiment is diagrammatically shown in Fig. 4. At t_0 subsidiary manometers 1 and 2 (left) and the

main manometer (H_0) are read. The left sampling tap is immediately turned in order to connect the first pair of sampling cups (c_0 and c'_0) with their manometers. At t_1 subsidiary manometers 3 and 4 (right) and the main manometer (H_1) are read. The right sampling tap is then turned to connect c_1 and c'_1 with their manometers. At t_2 subsidiary manometers 1 and 2 (their pressures, h_0 and h'_0, having in the meantime become stationary) and the main manometer (H_2) are read. The left tap is then turned once more to connect the next pair of sampling cups (c_2 and c'_2) with their manometers. At t_3 subsidiary manometers 3 and 4 (h_1 and h'_1) and the main manometer (H_3) are read and the right tap turned once more to connect the last pair of sampling cups (c_3 and c'_3) with their manometers. All readings of the main manometer (H_0, H_1, H_2 and H_3) have by now been taken. The remaining readings, h_2 and h'_2 (left), h_3 and h'_3 (right), are taken when the pressures in these manometers are constant.

In apparatus *B* the left inner key is turned at t_0, the right inner key at t_1, the left outer key at t_2 and the right key at t_3 (Fig. 5). The readings are taken in the way described for apparatus *A*.

CALIBRATION

The most accurate way of calibrating the apparatus is with mercury. In the main vessel the total volume has to be determined (including the eight sampling cups), and the separate volume of each sampling cup. The constants of the subsidiary manometers can also be obtained experimentally. It is not advisable to do so in the first instance if no previous experience of the handling of the apparatus has been gained. However, it may be useful to check the calibrated constants in the following ways.

Constants of the subsidiary manometers for the determination of gaseous carbon dioxide. The main vessel contains water and both rolls of filter paper in the subsidiary manometers on each side are wetted with water (no KOH to be included). A positive pressure is produced in the main manometer by a gas other than CO_2. This corresponds to the reading H_0. In this case $H_0 = H_1 = H_2 = H_3$. The left and right sampling keys are turned once.

$$k_j = \frac{H_j K_j c_j}{V_{a_j} h_j} \quad (j = 1 \text{ or } 2).$$

The manometer fluid in the subsidiary manometers is then levelled and the procedure continued.

$$k_j = \frac{H_j K_j c_j}{V_{a_j} h_j} \quad (j = 2 \text{ or } 3).$$

Constants of the bicarbonate-carbon dioxide manometers. A bicarbonate solution of known concentration is made up (approximately 50 μl. CO_2/ml.) and its concentration checked by acidifying in an ordinary manometer. The bicarbonate solution is pipetted into the main vessel and the lower outlet tubes filled with mercury and tartaric acid. After temperature equilibration, the sampling taps are turned as described. The subsidiary manometers are not levelled after the first readings left and right. If the known bicarbonate concentration/ml. is *A*,

$$k'_{j:CO_2} = \frac{Ac'_j}{h'_j} \quad (j = 1 \text{ or } 2),$$

$$k'_{2:CO_2} = \frac{Ac'_2 + (h'_0 k'_{0:CO_2})}{h'_2},$$

$$k'_{3:CO_2} = \frac{Ac'_3 + (h'_1 k'_{1:CO_2})}{h'_3}.$$

Protocols

Exp. 1. This concerned the influence of light, in air, on the metabolism of *Gastrophilus intestinalis* larvae. It was done with Mr L. Levenbook as part of a study of the influence of CO on the metabolism of *Gastrophilus intestinalis* larvae. The significance of the light effect on the ratio CO_2/O_2 is not discussed here. Apparatus: type *B*; temp., 37°; constants given in Table 4. The larvae rested on wetted filter paper.

Table 4. *Constants of apparatus used in Exp.* 1

	$k_0 = 0{\cdot}757$		
$K_{1:O_2} = 4{\cdot}4464$	$k_1 = 0{\cdot}8303$		
$K_{2:O_2} = 4{\cdot}30$	$k_2 = 0{\cdot}6441$		
$K_{3:O_2} = 4{\cdot}1472$	$k_3 = 0{\cdot}7360$		
A = 0·0985	**F** = 0·9387	**L** = 49·6421	**V** = 0·9831
B = 0·0915	**G** = 1·10	**M** = 43·8393	**X** = 0·9785
C = 0·10	**H** = 0·985	**N** = 44·7451	**Z** = 0·9776
D = 0·0948			
$\mathbf{E}_l$ = 0·4727			
$\mathbf{E}_r$ = 0·5501			

The main vessel contained in addition to the larvae a small metal boat, filled with water. The total volume of larvae, boat and water was adjusted to 5 ml. Wet weight of four larvae, 1·9 g.

Table 5. *Manometer readings, Exp.* 1

t (min.)	Experimental conditions	Main manometer		Subsidiary manometer, gaseous CO_2
		$H_0 = -0{\cdot}2$		$h_0 = -14{\cdot}1$
0–30	Light	$H_1 = +21{\cdot}2$	$\Delta H_1 = +21{\cdot}4$	$h_1 = -17{\cdot}5$
30–60	Dark	$H_2 = +5{\cdot}2$	$\Delta H_2 = -16{\cdot}0$	$h_2 = -45{\cdot}6$
60–90	Light	$H_3 = +35{\cdot}0$	$\Delta H_3 = +29{\cdot}8$	$h_3 = -51{\cdot}2$

The readings (Table 5) give the following relationships:

$$\text{I.}\quad x_{0:CO_2} = 0{\cdot}0985 \times -0{\cdot}2 + 14{\cdot}1 \times 0{\cdot}757 = 10{\cdot}66.$$

$$\text{II.}\quad x_{1:CO_2} = 0{\cdot}09147 \times 21{\cdot}2 + 17{\cdot}5 \times 0{\cdot}8303 = 16{\cdot}51.$$

$$\text{III.}\quad \Delta x_{1:CO_2} = 16{\cdot}51 - 0{\cdot}9387 \times 10{\cdot}66 = 6{\cdot}51.$$

$$\text{IV.}\quad x_{2:CO_2} = 0{\cdot}10 \times 5{\cdot}2 + 0{\cdot}4727 \times -14{\cdot}1 + 45{\cdot}6 \times 0{\cdot}6641 = 23{\cdot}24.$$

$$\text{V.}\quad \Delta x_{2:CO_2} = 23{\cdot}24 - 1{\cdot}10 \times 16{\cdot}51 = 5{\cdot}04.$$

$$\text{VI.}\quad x_{3:CO_2} = 0{\cdot}09481 \times 35{\cdot}0 + 0{\cdot}5501 \times -17{\cdot}5 + 51{\cdot}2 \times 0{\cdot}8303 = 31{\cdot}39.$$

$$\text{VII.}\quad \Delta x_{3:CO_2} = 31{\cdot}39 - 0{\cdot}985 \times 23{\cdot}24 = 8{\cdot}54.$$

$$\text{VIII}a.\quad \Delta X_{1:CO_2} = \frac{49{\cdot}6421}{1{\cdot}9} \times 6{\cdot}51 = 170.$$

$$\text{IX}a.\quad \Delta X_{2:CO_2} = \frac{43{\cdot}8393}{1{\cdot}9} \times 5{\cdot}04 = 116{\cdot}5.$$

$$\text{X}a.\quad \Delta X_{3:CO_2} = \frac{44{\cdot}7451}{1{\cdot}9} \times 8{\cdot}54 = 200{\cdot}3.$$

$$\text{XXI}a.\quad \Delta X_{1:O_2} = \frac{21{\cdot}4}{1{\cdot}9} \times 4{\cdot}4464 - 0{\cdot}9831 \times 170 = -117.$$

$$\text{XXII}a.\quad \Delta X_{2:O_2} = \frac{-16}{1{\cdot}9} \times 4{\cdot}30 - 0{\cdot}9785 \times 116{\cdot}5 = -150{\cdot}2.$$

$$\text{XXIII}a.\quad \Delta X_{3:O_2} = \frac{29{\cdot}8}{1{\cdot}9} \times 4{\cdot}1472 - 0{\cdot}9776 \times 200{\cdot}3 = -131.$$

The results are given in Table 6.

Table 6. *Results of Exp.* 1

(q'_{O_2} and q'_{CO_2} represent O_2 uptake and CO_2 production/1 g. larvae/hr.)

t (min.)	Experimental conditions	q'_{O_2}	q'_{CO_2}	$\frac{q'_{CO_2}}{q'_{O_2}}$
0–30	Light	234	340	1·45
30–60	Dark	300·4	233	0·775
60–90	Light	262	400·6	1·53

Exp. 2. This concerned the metabolism of 4 sheep retinae in Ringer-bicarbonate with 0·2 % glucose and 5 %CO_2/95 %O_2 at 38°. Apparatus: type *B*; constants given in Table 7. Manometer fluids: paraffin (*d*, 0·788) in main manometer and in subsidiary manometers for determination of gaseous CO_2; α-bromonaphthalene (*d*, 1·4875) in subsidiary manometers for determination of bicarbonate-CO_2. Extensions were used on the lower outlet tubes (see Fig. 3, *J*).

Owing to large pressure changes occurring in the subsidiary manometers for the determination of the gaseous CO_2 at t_2 and t_3, they were opened and levelled after the determinations of h_0 and h_1. The expressions E_1h_0 and E_rh_1 in equations IV and VI, therefore, become zero and are omitted. Dry weight of retinae, 101 mg.

Table 7. *Constants of the apparatus used in Exp.* 2

$V_{F_1} = 24{\cdot}0745$	$K_{1.O_2} = 3{\cdot}08$	$k_0 = 0{\cdot}757$	$k'_{0:CO_2} = 3{\cdot}92$
$V_{F_2} = 23{\cdot}2613$	$K_{2:O_2} = 2{\cdot}996$	$k_1 = 0{\cdot}8303$	$k'_{0:CO_2} = 3{\cdot}97$
$V_{F_3} = 22{\cdot}4792$	$K_{3:O_2} = 2{\cdot}899$	$k_2 = 0{\cdot}6641$	$k'_{0:CO_2} = 3{\cdot}99$
		$k_3 = 0{\cdot}7360$	$k'_{3:CO_2} = 4{\cdot}07$

A = 0·1009	**F** = 0·9387	**L** = 43·4279
B = 0·09488	**G** = 1·10	**M** = 38·8585
C = 0·1026	**H** = 0·985	**N** = 37·5445
D = 0·1029		
O = 4·2562	**V** = 0·7486	
P = 4·3398	**X** = 0·7496	
Q = 4·6820	**Z** = 0·7499	
R = 4·5999		
S = 4·6504		
T = 4·5361		

Table 8. *Manometer readings, Exp.* 2

t (min.)	Main manometer		Subsidiary manometers: Gaseous CO_2	Subsidiary manometers: Bicarbonate CO_2
	$H_0 = -127{\cdot}4$		$h_0 = -116$	$h_0' = +125$
0–20	$H_1 = -37{\cdot}2$	$\Delta H_1 = +90{\cdot}2$	$h_1 = -127$	$h_1' = +116{\cdot}6$
20–40	$H_2 = +7{\cdot}8$	$\Delta H_2 = +45{\cdot}0$	$h_2 = -212{\cdot}5$	$h_2' = +227{\cdot}3$
40–60	$H_3 = -34{\cdot}5$	$\Delta H_3 = +26{\cdot}7$	$h_3 = -210{\cdot}3$	$h_3' = +216{\cdot}6$

The readings (Table 8) give the following relationships:

$$\text{I.} \quad x_{0:CO_2} = 0{\cdot}1009 \times -128 + 116 \times 0{\cdot}757 = 75.$$
$$\text{II.} \quad x_{1:CO_2} = 0{\cdot}09488 \times -37{\cdot}2 + 127 \times 8303 = 101{\cdot}9.$$
$$\text{III.} \quad \Delta x_{1:CO_2} = 101{\cdot}9 - 0{\cdot}9387 \times 75 = 31{\cdot}5.$$
$$\text{IV.} \quad x_{2:CO_2} = 0{\cdot}1026 \times 7{\cdot}8 + 212{\cdot}5 \times 0{\cdot}6641 = 142.$$
$$\text{V.} \quad \Delta x_{2:CO_2} = 143{\cdot}3 - 1{\cdot}1 \times 101{\cdot}9 = 30{\cdot}0.$$
$$\text{VI.} \quad x_{3:CO_2} = 0{\cdot}1029 \times 34{\cdot}5 + 210{\cdot}3 \times 0{\cdot}7360 = 158{\cdot}6.$$
$$\text{VII.} \quad \Delta x_{3:CO_2} = 158{\cdot}6 - 0{\cdot}985 \times 142 = 18{\cdot}6.$$

$$\text{VIII}a. \quad \Delta X_{1:CO_2} = \frac{43{\cdot}4279}{101} \times 31{\cdot}5 = 13{\cdot}8.$$

$$\text{IX}a. \quad \Delta X_{2:CO_2} = \frac{38{\cdot}8585}{101} \times 30{\cdot}0 = 11{\cdot}6.$$

$$\text{X}a. \quad \Delta X_{3:CO_2} = \frac{37{\cdot}5445}{101} \times 18{\cdot}6 = 6{\cdot}95.$$

$$\text{XI.} \quad X_{0:B} = 125 \times 4{\cdot}2562 = 532.$$
$$\text{XII.} \quad X_{1:B} = 116{\cdot}6 \times 4{\cdot}3388 = 506.$$
$$\text{XIII.} \quad X_{2:B} = 227{\cdot}3 \times 4{\cdot}6820 - 125 \times 4{\cdot}5999 = 489{\cdot}3.$$
$$\text{XIV.} \quad X_{3:B} = 216{\cdot}6 \times 4{\cdot}6504 - 116{\cdot}6 \times 4{\cdot}5361 = 478{\cdot}3.$$

$$\text{XV}a. \quad \Delta X_{1:G} = \frac{24{\cdot}0745}{101} \times (532\text{–}506) = 6{\cdot}23.$$

$$\text{XVI}a. \quad \Delta X_{2:G} = \frac{23{\cdot}26125}{101} \times (506\text{–}489{\cdot}3) = 3{\cdot}84.$$

$$\text{XVII}a. \quad \Delta X_{3:G} = \frac{22{\cdot}4791}{101} \times (489{\cdot}3\text{–}478{\cdot}3) = 2{\cdot}48.$$

$$\text{XVIII}a. \quad \Delta X^{\text{resp.}}_{1\,CO_2} = 13{\cdot}8 - 6{\cdot}23 = 7{\cdot}57.$$
$$\text{XIX}a. \quad \Delta X^{\text{resp.}}_{2\,CO_2} = 11{\cdot}6 - 3{\cdot}84 = 7{\cdot}76.$$
$$\text{XX}a. \quad \Delta X^{\text{resp.}}_{3\,CO_2} = 6{\cdot}95 - 2{\cdot}48 = 4{\cdot}47.$$

$$\text{XXI}a. \quad \Delta X_{1:O_2} = \frac{90{\cdot}2}{101} \times 3{\cdot}08 - 0{\cdot}7486 \times 13{\cdot}8 = 7{\cdot}57.$$

$$\text{XXII}a. \quad \Delta X_{2:O_2} = \frac{45{\cdot}0}{101} \times 2{\cdot}9962 - 0{\cdot}7496 \times 12{\cdot}0 = 7{\cdot}49.$$

$$\text{XXIII}a. \quad \Delta X_{3:O_2} = \frac{26{\cdot}7}{101} \times 2{\cdot}899 - 0{\cdot}7499 \times 6{\cdot}45 = 4{\cdot}44.$$

Table 9. *Results of Exp.* 2

(Q_{O_2}, $Q^{\text{resp.}}_{CO_2}$, Q_G, metabolic quotients of O_2, respiratory CO_2 and glucose are in μl./mg. dry wt./hr.)

t (min.)	Q_{O_2}	$Q^{\text{resp.}}_{CO_2}$	Q_G	R.Q.
0–20	22·7	22·7	18·7	1·0
20–40	22·5	23·3	11·5	1·04
40–60	13·3	13·4	7·5	1·0

Results are given in Table 9. The figures for O_2 uptake and total CO_2 production agree closely with those obtained when using the indirect method of Warburg (1924, 1925).

SUMMARY

1. The existing manometric methods of measuring the respiratory quotient allow only one determination to be made during one experimental period. So far it has not been possible to measure changes of R.Q. with time, or after alterations of the experimental conditions during the experiment. Such changes occur on addition of substrates, inhibitors, catalysts, drugs, or on fertilization.

2. A new manometric apparatus, which allows such changes to be measured on a single sample of biological material, has been made.

3. The method involves taking samples of the liquid and gaseous phases from the main vessel. Samples are taken at the beginning of the experiment and at three convenient times during the experiment, which is unaffected by the sampling procedure. The CO_2 and bicarbonate contents of the samples are

determined in subsidiary manometers. These, together with the readings of the main manometer at the times of sampling, enable the O_2 uptake, and the production of respiratory CO_2 and acid to be independently calculated for each interval between sampling. In this way changes with time in the rates of O_2 uptake, total CO_2 production and of R.Q. can be obtained.

4. Two forms of the apparatus have been designed and are described. The theory of the method is developed and a simplified way of computing the results is given.

The instruments were made by Messrs W. G. Flaig and Sons, Waterloo Road, London, N.W. 2.

REFERENCES

Dickens, F. & Simer, F. (1930). *Biochem. J.* **24**, 905.
Dickens, F. & Simer, F. (1931). *Biochem. J.* **25**, 973.
Dixon, M. & Keilin, D. (1933). *Biochem. J.* **27**, 86.
Laser, H. & Rothschild, Lord (1939). *Proc. Roy. Soc.* B, **126**, 539.
Sumerson, W. H. (1939). *J. biol. Chem.* **131**, 579.
Warburg, O. (1924). *Biochem. Z.* **152**, 51.
Warburg, O. (1925). *Biochem. Z.* **164**, 481.

Carotenoids, Vitamin A and 7-Dehydrosteroid in the Frog (*Rana temporaria*)

By R. A. MORTON and D. G. ROSEN
Department of Biochemistry, University of Liverpool

(*Received* 2 *March* 1949)

Understanding of the biochemistry of carotenoids is rudimentary except as to the part played in the animal economy by a few of them as precursors of vitamin A. In a wider sense the animal biochemistry is complicated by species differences. Thus some animals absorb and retain so little of either the hydrocarbons or their hydroxylated derivatives as to suggest that they are not needed, whilst other species absorb relatively large quantities of different carotenoids selectively and retain them distributed over various organs. For the carotenes it could be argued that they are only absorbed as such when the capacity of enzyme systems in the gut to transform them is exceeded, and that the pigments, of no special value to the body, are slowly disposed of. Such an argument, however, neglects the regular occurrence of carotenoids in eyes, yellow bone marrow and corpora lutea and is of little value in respect to the assimilation and metabolism of hydroxylated carotenoids like lutein and zeaxanthin.

As a subject for detailed study the frog has several special advantages; carotenoids (including both β-carotene and lutein) are widely distributed in the body; the species can be studied through the life cycle, as embryos, as tadpoles and as immature and mature frogs; there is a marked seasonal cycle in both sexes and the reproductive phase entails a heavy drain on body reserves, especially in the female. Moreover, as an amphibian, the frog provides a link between work on mammals and fishes. There are, therefore, numerous factors potentially capable of correlation with data on the distribution of carotenoids so as to lead to hypotheses concerning the functions of these substances.

For 10–11 weeks after using up the residual yolk the tadpole is mainly vegetarian, but when metamorphosis is complete, frogs become mainly carnivorous. Adult frogs build up large fat reserves in early summer, and from June to August the testes or ovaries enlarge considerably. The testes then shrink and during the next few months vary little in size until the spermatozoa become fully ripe; the ovaries go on developing, but after spawning in March the shrunken oviducts are orange in contrast to their earlier paleness. The corpora adiposa of Amphibia are associated with the gonads; in the frog they vary from strands of orange tissue to large cream-coloured lobes. After surgical removal of one fat body the ovary on the operated side remains small compared with the other. According to March (1937) sexually mature frogs have a body length (snout-cloaca) of about 4·8 cm. and weigh about 10 g. Applied with caution, body length is a guide to age.

FAT SOLUBLE SUBSTANCES IN THE FROG

Earlier work on carotenoids and vitamin A. Lönnberg (1929) reported the presence of a 'carotene-like pigment' in the skin (*Rana temporaria* and *R. esculenta*). Dietel (1933) noted the presence of lipochrome in skin, ovaries and liver, but thought it consisted wholly of carotene. Manunta (1934) found 'xanthophyll' and much carotene in the skin of *R. esculenta*. Rand (1935) observed vitamin A to be present in frog and toad livers with lipochrome pigments detectable in skin, liver, ovaries and eggs, oviducts, testes, kidneys,

lungs, spleen and fat bodies. He did not discriminate between free 'xanthophyll' and esterified 'xanthophylls' so that his analyses are incomplete. He used too few frogs for his work to be more than exploratory. Brunner & Stein (1935), using phase partition and chromatography, concluded that, in *R. esculenta*, β-carotene and lutein (free or esterified) were the only carotenoid pigments present. In the fat bodies, liver and skin, the 'xanthophyll' was all ester form, but free lutein also occurred in the ovaries. Zechmeister & Tuzson (1936), starting from 491 frog livers, prepared crystalline specimens of liver carotenoids. The skin, ovaries, fat bodies and livers of fourteen females (*R. esculenta*) were quantitatively analysed by chromatographic and spectroscopic methods. Three fractions were separated: (*a*) epiphasic pigment, (*b*) free hypophasic pigment, and (*c*) esterified hypophasic pigment, the latter always predominating; (*a*) consisted of α- and β-carotene, the β-isomeride preponderating; (*b*) was a mixture of lutein and zeaxanthin. Many unidentified zones were observed during the chromatography, but it is not clear to what extent the substances responsible were metabolic products or *in vitro* artefacts. Other workers include Bartz & Schmitt (1936), van Eekelen (1934), Wald (1935), Ackermann (1938), Gillam (1938) and Lederer & Rathmann (1938).

Origin of ingested carotenoids. Relatively little is known about the carotenoids of the various species of insects, snails and worms which form the diet of frogs, although β-carotene and lutein probably preponderate. Judging from the contents of the alimentary tracts of frogs (in which chlorophyll is always present) a large proportion of the ingested carotenoid is in the form of plant material unabsorbed from the digestive tracts of insects eaten. From the point of view of carotenoid intake the frog is indirectly vegetarian to the extent that it makes use of plant pigments which have not previously entered the body proper of animals.

GENERAL PLAN OF THE PRESENT WORK

Neither the design of the experiment as a whole nor the interpretation of data is easy. If it had been possible to work on 600 frogs individually rather than in batches of twenty, the plan could have been different and the data obtained could have been treated statistically. Many of the analyses, however, would have been impossible on organs from single frogs. The choice lay between making a general survey with its attendant limitations and greatly restricting the scope of the work. On balance, taking into account the paucity of information in the field, it seemed better to secure the analytical data which time permitted on samples convenient to deal with, than to select particular variables for study on larger numbers of frogs.

EXPERIMENTAL

Animals

The frogs were obtained from a commercial supplier (Norwich district) and delivered at monthly intervals. Each batch consisted of about twenty males and twenty females. The sexes were segregated on arrival. The frogs were weighed, killed by pithing, and dissected. The various organs, livers, kidneys, fat bodies, etc. from twenty animals of the same sex were combined and weighed. Each sample was then analysed for total fat and the fat in turn analysed quantitatively for as many of the following as seemed appropriate in each case: (*a*) vitamin A (free and esterified), (*b*) total carotenoids, (*c*) carotene, (*d*) 'xanthophyll', (*e*) free 'xanthophyll', mono-ester and di-ester. Within a batch, the work on one sex was completed before beginning on the other.

Batches of frogs were obtained from late March to December in 1946 and 1947 and some in 1948. The only part of the annual cycle which was not studied was the period of actual hibernation. Ova were obtained at all stages of maturity and tadpoles at all stages between the fertilized egg and the young frog. At various periods one of us (D.G.R.) collected animals from Hillside, near Southport, Lancashire, for additional tests. These included the tadpoles and young frogs (*R. temporaria*) and tadpoles of the natterjack toad (*Bufo calamita*).

Chemical methods

Outline. Specimens of tissue were ground with sand and anhydrous Na_2SO_4 and extracted with ether. The solvent was removed and the lipid made up to volume with light petroleum. Measured volumes were taken for the determination of vitamin A, carotenoids and fat. Chromatographic methods were used to separate the components and the quantitative determinations were based on data obtained with the Beckman photoelectric spectrophotometer. Exposure of extracts to light was avoided so far as possible. The extracts were not subjected to heat treatment at any stage, except in removing solvent in the fat determination.

Material for analysis. The frogs, which had been kept in tanks, supplied with water but no food, were roughly dried with blotting paper before weighing and dissecting. After weighing, the frogs were killed and dissected as quickly as possible. Before the viscera were weighed, adhering coelomic and other fluids and clotted blood were removed; the weighings were made without delay.

Solvents. Diethyl ether was always freshly distilled over a little reduced Fe before use. Light petroleum (b.p. 40–60°) must be practically free from 'polar' impurities; the commercial product is variable and must either be rigorously purified or selected for chromatography. A consignment of good quality petroleum was reserved for this study. The acetone was of ordinary reagent quality and the $CHCl_3$ of commercial B.P. quality. Ethanol and *cyclo*hexane were specially purified for spectroscopy.

Total fat. The portion of the ethereal extract which was soluble in light petroleum was weighed after dehydration with ethanol and recorded as 'total fat'.

Extraction. The tissue was quickly ground, a little at a time, in a mortar with about five times its weight of anhydrous Na_2SO_4 to which fine acid-washed silver sand was added. Tadpoles were treated similarly without dissection. The well-ground material was transferred to a beaker and covered with ether. After standing 1–2 hr. in the dark, the yellow extract was decanted through a sintered glass filter (G4) under suction. The solid residue was then transferred to the filter cup and washed with successive portions of ether until the filtrate became colourless. The solid was then returned to the beaker and left to stand under ether for

0·5–1 hr. and filtered. A third extraction, during which the ether was boiled for a short time, yielded no more colour but removed residual vitamin A. In some cases the finely ground tissues were allowed to stand overnight under ether in the dark in a corked flask. The solution was decanted on the filter and the residue washed with ether until no more pigment came through. Skin, muscle, lungs, oviducts, oesophagus, stomach and intestines were extracted overnight, other tissues as quickly as possible. In all cases, the ether was removed by distillation under reduced pressure at room temperature. The residue was dissolved in light petroleum and made up to volume. A small amount of anhydrous Na_2SO_4 was added if the solution was not perfectly clear. A measured portion of the extract was used for direct estimation of vitamin A, the light petroleum being removed and replaced by a suitable volume of *cyclo*hexane.

Chromatographic separation

Commercial bone meal was 'defatted' by means of boiling acetone. The fraction which then passed through a 120 British Standards Specification sieve was used for chromatography. When such material is stirred with acetone, the smallest particles do not settle readily, and if the liquid is decanted the 'fines' can be removed. This is an advantage as otherwise acetone eluates may be cloudy. Alumina (Savory and Moore) which had been in stock in the laboratory for some years and had become rather weakly adsorbent proved well suited to the separation of carotenoids. Type 'O' alumina (Peter Spence and Co.) could be reproducibly weakened by stirring in definite proportions of water.

A chromatographic tube of 18 mm. internal diameter was used throughout this work. A column of bone meal 3 cm. in height sufficed for the separation of vitamin A ester from the free vitamin and a 4 cm. column for carotenoid separation. The adsorbent was poured dry into the tube, light petroleum was added and the bone meal stirred with a long thin metal rod to liberate trapped air bubbles. Strong suction was applied, but from then onwards the column was not allowed to run 'dry'. The initial adsorption of solutes was effected slowly under gravity alone. Development and elution were more rapid at 120–150 drops/min. under suction. Small volumes of eluate were aimed at and colourless intermediate fractions obtained in carotenoid analyses were discarded. Vitamin A esters were eluted completely by 100 ml. of light petroleum and the free vitamin by means of a little acetone. Solvents were removed under reduced pressure and the residues made up to suitable volume in *cyclo*hexane.

Carotene (in some cases contaminated with esterified 'xanthophyll') was carried through the bone-meal column by not more than 25 ml. of light petroleum. A measured portion of this eluate was put through a similar adsorption tube containing a 4 cm. column of alumina; here 30 ml. of light petroleum carried down all the carotene and the esterified 'xanthophyll' was washed through with acetone (10–20 ml.). The free 'xanthophyll' retained on the bone-meal column was eluted with acetone. Throughout this paper 'xanthophyll' refers to a fraction consisting mainly of lutein, but possibly containing some zeaxanthin and a little kryptoxanthin.

For a more complete separation of fractions containing little vitamin A a fresh portion of extract was adsorbed on alumina, the carotenes eluted by means of light petroleum, the 'xanthophyll' di-esters by 1% of acetone in light petroleum (v/v) and the mono-esters by 8% of acetone in light petroleum (v/v). In some cases the 'xanthophyll' fraction from bone meal was eluted with acetone, the solvent completely removed and replaced by light petroleum and the solution chromatographed on alumina. When necessary, di-esters and mono-esters can be selectively eluted from bone meal by means of 2% (v/v) and 8% (v/v) $CHCl_3$ in light petroleum, respectively. The final fractions were made up to volumes suitable for spectroscopic examination. In replacing one solvent by another heat treatment was avoided. The visible spectra of the three 'xanthophyll' fractions are reproduced in Fig. 1. If the extracts were saponified before chromatography, the 'xanthophyll' fraction was substantially homogeneous.

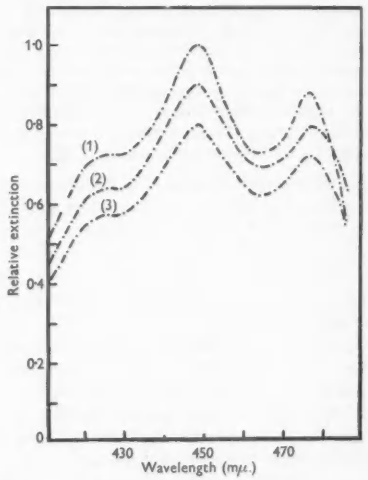


Fig. 1. Visible absorption spectra in acetone of the three forms of 'xanthophyll': (1) free xanthophyll; (2) xanthophyll di-ester; (3) xanthophyll mono-ester.

Spectroscopic determinations

Vitamin A. Vitamin A was determined by the intensity of absorption at 328 mμ. and the gross $E^{1\%}_{1\,cm.}$ values were corrected for irrelevant absorption by the method of Morton & Stubbs (1946). The separation of esterified vitamin A from the free alcohol was carried out by the method of Glover, Goodwin & Morton (1947) using bone meal as chromatographic adsorbent. As a rule the amount of free vitamin A was small and was best determined by the difference between the total (corrected) and ester (corrected).

Carotenoids. The spectra of nearly all the fractions agreed very closely with those of the corresponding purified carotenoids from other sources when the criteria adopted were (*a*) wavelengths of maximum absorption in specified solvents, and (*b*) relative intensities of absorption at maxima and minima (Fig. 2). However, certain carotenoid fractions, from intestines and eyes, for example, exhibited irrelevant absorption. This was particularly noticeable in the lipids from frog eyes (Fig. 3). The colour is due mainly to 'xanthophyll' and the curve may be corrected for irrelevant

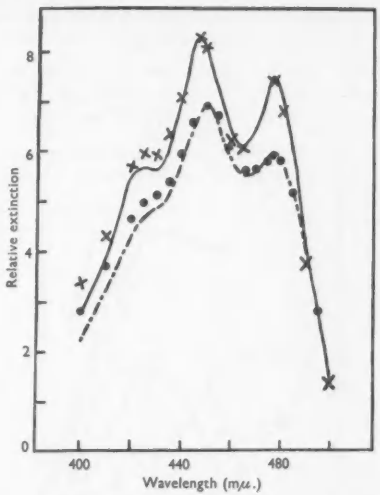


Fig. 2. Comparison of visible absorption spectra of pure carotenoids and frog carotenoid extracts: ———, lutein in ethanol; × × × ×, frog xanthophyll in ethanol; –·–·–, β-carotene in *n*-hexane; ·····, frog carotene in *n*-hexane.

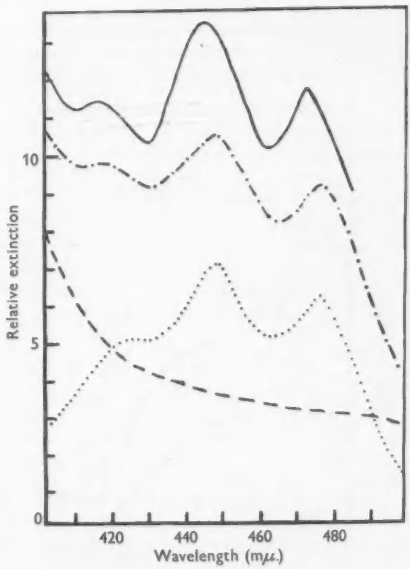


Fig. 3. Visible absorption spectra of frog-eye extracts: ———, whole extract (light petroleum 40–60°); –·–·–, total 'xanthophyll' (acetone); ·····, corrected 'xanthophyll'; – – – –, irrelevant absorption.

absorption by the general method of Morton & Stubbs (1946) on the basis of the extent of distortion of the mean 'xanthophyll' curve (see Fig. 2) of extracts of other organs. The relative absorptions at the three wavelengths 448·5, 463 and 476 mμ. are $E=1$, 0·724, 0·881 respectively, and $E_{448\cdot5}$ mμ. (corr.) $=E_1\times2\cdot216-E_2\times4\cdot688+E_3\times2\cdot472$, where E_1, E_2 and E_3 are observed values at the three wavelengths, respectively.

It was sometimes necessary to examine solutions so dilute that the photoelectric photometer was perforce used outside the recommended range of extinctions. The instrument was therefore calibrated over the range $E=0\cdot01$ to $E=1\cdot0$ using carotene in light petroleum and 'xanthophyll' esters in acetone or light petroleum. When readings below $E=0\cdot2$ were obtained with frog extracts, corrections derived from the calibration curves were applied. In the cases of mono-esterified and free 'xanthophyll' fractions in acetone similar calibration demonstrated that no such correction was necessary. The data in Table 1 were used as a basis for calculations.

Table 1. *Spectrophotometric data on carotenoids, used in the analytical work*

Substance	$\lambda_{max.}$ (mμ.)	$E^{1\%}_{1\,cm.}$	Solvent	Remarks
β-Carotene	449·5	2550	Light petroleum	(*a*)
Lutein	446·5	2550	Ethanol	(*b*)
Vitamin A	328	1700	*Cyclo*Hexane	(*a*)
Frog 'xanthophyll'	448·5	2640	Acetone	(*c*)
Frog 'xanthophyll' di-esters	448	2550	Acetone	(*c*)
Frog 'xanthophyll' mono-esters	448	2600	Acetone	(*c*)

(*a*) Determinations in this laboratory; (*b*) Zscheile, White, Beadle & Roach (1942); (*c*) values determined in the course of the present work relative to $E^{1\%}_{1\,cm.}$ 2550 for pure free lutein.

If, in determining the 'total carotenoid' per frog in a given type of tissue, x frogs were used to provide an extract in y ml. and the solution had to be diluted by a factor z so as to bring $E_{max.}$ for a 1 cm. layer to a convenient figure; then

$$\frac{Ez}{100x/y}=\frac{Eyz}{100x}=Q.$$

The use of Q avoids the difficulty that 'total carotenoid' is a mixture of components in which neither $\lambda_{max.}$ nor $E^{1\%}_{1\,cm.}$ is necessarily exactly the same. Since, however, $E^{1\%}_{1\,cm.}$ at the visible maximum is of the order 2500, i.e. E is about 2·5 for a 1 cm. layer of solution of concentration 1 mg./100 ml., $Eyz/250x$ is the weight of solute per frog in mg., so that roughly $0\cdot4Q$ = mg. total carotenoid/frog.

In this investigation the number of analyses was necessarily very large and the preparation of material time-consuming. In choosing methods the aim was the greatest accuracy compatible with speed. It was found to be essential to complete the tests on livers, kidneys and eyes, so far as vitamin A was concerned, in one working day; standing overnight might have caused a loss of as much as 40% of the vitamin. The intact organs could, however, be kept at 0° without appreciable loss (Table 2). The method of decomposing the tissue by boiling with ethanolic KOH and testing the non-saponifiable extract was tried side by side

with the procedure already described, and was found to be attended by significant losses or isomerization of carotenoids (Table 3).

Table 2. *Stability of carotenoids in frog tissues stored at 0° after dissection*

Tissue	Period of storage (days)	Total carotenoid $E^{1\%}_{1\,cm.}$ *	Total vitamin A $E^{1\%}_{1\,cm.}$ (corr.)*
Liver	0	0·0976	0·117
	1	0·0998	0·115
	3	0·0968	0·113
Kidney	0	Not tested	0·543
	1	Not tested	0·557
Muscle	0	0·00487	Nil
	3	0·00482	Nil

* Calculated on wet wt. of tissue.

Table 3. *Comparison of analyses for carotenoids and vitamin A carried out on (a) ether-extractable lipid and (b) non-saponifiable ether extract after treating tissue with ethanolic* KOH

Tissue	Total carotenoid $E^{1\%}_{1\,cm.}$ (a)	Total carotenoid $E^{1\%}_{1\,cm.}$ (b)	Vitamin A $E^{1\%}_{1\,cm.}$ (corr.)* (a)	Vitamin A $E^{1\%}_{1\,cm.}$ (corr.)* (b)
Cock liver	0·008	0·0072	Not measured	
Horse liver	0·0192	0·0179	0·248	0·263
Hare liver	Negligibly small		0·034	0·035
Frog skin	0·031	0·030	Negligibly small	
Frog liver	0·080	0·069	0·105	0·107
Frog liver	0·227	0·175	0·278	0·243
Frog testes	0·053	0·046	Negligibly small	

* Calculated by the procedure of Morton & Stubbs (1946).

Samples of frog carotenoid fractions with accompanying lipids were used to ascertain the magnitude of manipulative losses in the chromatographic procedures. Over the relevant range, recoveries of 97–99% were obtained. Similar tests with free and combined vitamin A provided equally good recoveries.

The magnitude of the correction for irrelevant absorption at 328 mμ. in tests on liver extracts is illustrated in Fig. 4, and on extracts from eyes and kidneys in Fig. 5. The full lines in Fig. 4 show irrelevant absorption at its greatest in the present series (in all there were three such cases); the broken-line curves illustrate the normal case in which the irrelevant contribution is about 20% (thirty cases). The carotenoid contribution amounted at 328 mμ. to only 3–4% of the total absorption. The need for correction at 328 mμ. is much more obvious in the extracts from eyes and kidneys, but the validity of corrected estimates for vitamin A content was confirmed using the $SbCl_3$ colour test.

When the ultraviolet absorption of an extract suggested the absence of vitamin A, the colour test was used to provide confirmation.

RESULTS AND DISCUSSION

The experimental findings are recorded in tables and figures.

Lipochromes and vitamin A in tadpoles and young frogs

Presence of chlorophyll. The absorption spectra of chromatographic fractions containing 'xanthophylls' show some chlorophyll to be present also.

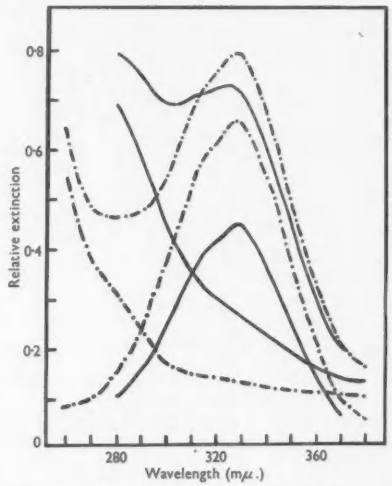


Fig. 4. Ultraviolet absorption spectra of liver extracts illustrating the magnitude of the correction: –·–·–, typical spectrum; ———, atypical spectrum. In each set the top curve is observed, the middle curve corrected and the lowest curve represents irrelevant absorption.

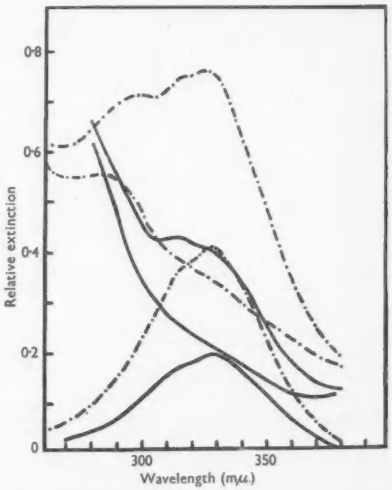


Fig. 5. Typical ultraviolet absorption spectra of eye (–·–·–) and kidney (———) extracts, showing gross, corrected and irrelevant absorption.

Thus, tadpole extracts exhibit maxima at 662–664, 615 and 432–434 mμ.; $E_{662\ m\mu.}/E_{615\ m\mu.} = 4{\cdot}2$. Harris & Zscheile (1943) give for chlorophyll-*a* $\lambda_{max.}$ 661·5, 615·5 and 431·5 mμ.; $E_{661{\cdot}5\ m\mu.}/E_{615{\cdot}5\ m\mu.} = 6{\cdot}3$. The low value for the ratio of the intensities of the two chlorophyll bands (Figs. 6 and 7) suggests the presence of chlorophyll-*b*. By applying the method of Comar & Zscheile (1942) to a fresh ether extract of tadpoles, chlorophyll-*a* was found to preponderate over chlorophyll-*b* by a factor of 7·5 to 1. The observed absorption in the region 400–500 mμ. is therefore a summation of contributions from 'xanthophyll' and 'chlorophyll'. The absorption in the region 600–700 mμ. is due solely to the latter, and from the absorption curve for pure chlorophyll-*a*, the curve from the red region can be continued through to the violet using the following relative *E* values:

λ	*662*	400	412	420	430
E	0·976	0·7	0·913	0·875	1·218
λ	*432*	440	450	460 mμ.	
E	1·225	0·595	0·074	0·018	

The 'xanthophyll' contribution in tadpole extracts is then obtained by difference (Fig. 6). In two samples, the 'chlorophyll' and 'xanthophyll' were separated by chromatography on weakened alumina and the validity of the difference method confirmed.

In extracts of natterjack tadpoles the chlorophyll contribution was computed in a similar manner, but an additional maximum near 420 mμ. due to the presence of an artefact was observed when the 'xanthophyll' contribution was obtained by difference. This substance, which showed a maximum at 412 mμ., was probably a degradation product of chlorophyll; it was easily removed by adsorption on alumina and the anomalous 'xanthophyll' absorption was corrected by subtraction (Fig. 7). Carotenoids are decomposed by exposure to light in the presence of chlorophyll and oxygen (Pepkowitz, 1944); it is necessary, therefore, to protect the extracts as much as possible. Vitamin A in traces is very difficult to detect in such mixtures, and the only way in which its presence was demonstrated in natterjack tadpoles was by the antimony trichloride colour test applied to extracts from the dissected eyes.

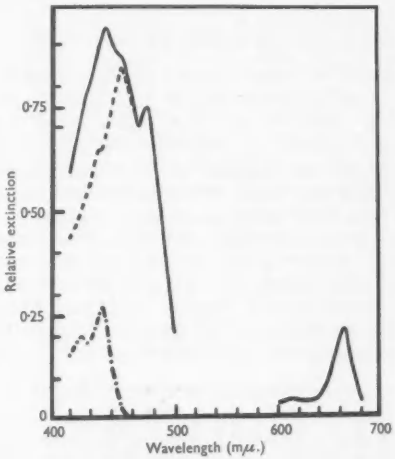


Fig. 6. Visible absorption spectra of frog tadpole 'xanthophyll' in acetone: ———, 'xanthophyll' from chromatogram; –·–·–, computed chlorophyll contribution; - - - - -, xanthophyll contribution.

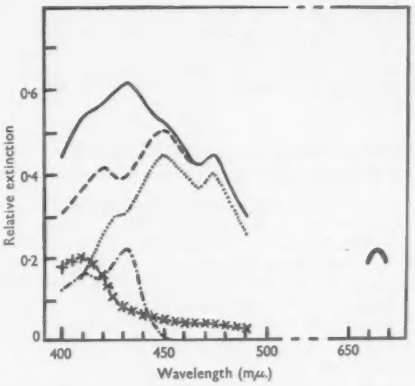


Fig. 7. Visible absorption spectra of natterjack tadpole 'xanthophyll' in acetone: ———, 'xanthophyll' from chromatogram; –·–·–, computed chlorophyll contribution; - - - - -, absorption of 'xanthophyll' plus artefact; ×–×–×, artefact contribution; ....., 'xanthophyll' contribution.

Occurrence of carotenoids. The number of tadpoles used was ample for the methods applied, and the data obtained are shown in Tables 4–6. The tadpoles were grouped in accordance with the stages of development *A–H* which can be fitted into an approximate time scale (see Fig. 8).

Comment. The chlorophyll is almost certainly all present in the digestive tract. The tadpoles were kept for 3 days in captivity after collection, but although they were active and faecal excretion copious, it was found that those collected at stage *A* had to be kept for 9 days before chlorophyll disappeared. In tadpoles caught at different ages the chlorophyll content is considerable in stages *B*, *C* and *D*, less in *E*, very low in *F* and negligible in *G*. This suggests a decrease in food intake which is borne out by the weights and the carotenoid analyses (Table 4).

Table 4. *Carotenoids in tadpoles of* Rana temporaria *at various stages of development*

(Chlorophyll was present in *A–F*, not detected in *G* and *H*.)

Stage	Date and number used (in brackets)	Average wt. (g.)	Carotene/ tadpole (μg.)	'Xantho-phyll'/ tadpole (μg.)	Ratio 'xantho-phyll'/ carotene
A, young tadpoles, 10–20 mm.	2. v. 47 (312)	0·049	0·012	0·11	9·7
B, tadpoles, 20–30 mm.	2. v. 47 (236)	0·12	0·033	0·30	9·1
C, tadpoles, 30–40 mm., rear leg just seen	20. v. 47 (75)	0·32	0·31	1·78	5·6
D, rear legs less than 4 mm.	4. vi. 47 (82)	0·22	0·27	0·93	3·4
E, rear legs greater than 4 mm.	4. vi. 47 (52)	0·25	0·40	1·21	3·0
F, fore limbs formed, some free	4. vi. 47 (73)	0·22	0·25	0·92	3·7
G, all four limbs well formed, long tails persisting	10. vi. 47 (88)	0·19	0·23	0·72	3·1
H, small frogs, tails almost resorbed	10. vi. 47 (93)	0·15	0·25	0·76	2·8

Table 5. *Rate of disappearance of carotene and 'xanthophyll' during metamorphosis of tadpoles*

	Average amounts of pigments (μg./tadpole)		Loss (%)
	Stages *C*, *D* and *E**	Stages *F*, *G* and *H*†	
Carotene	0·33	0·25	24
'Xanthophyll'	1·31	0·80	38

* Feeding freely. † Fasting.

Table 6. *Rate of disappearance of carotene and 'xanthophyll' from young tadpoles caught at stage A and kept in captivity without food*

	Pigments expressed as (μg./tadpole)		Loss (%)
	Tested 3 days after capture	Tested 9 days after capture*	(approx.)
Carotene	0·0116	0·0093	20
'Xanthophyll'	0·112	0·087	22

* Chlorophyll absent.

The amount of carotenoid in the original eggs is less than one quarter of that present in active tadpoles. Now the ratio of 'xanthophylls' to carotenes in the plant world, although variable, is only very rarely outside the range 3–10. In young tadpoles the ratio is initially about 9 and falls to about 3 at the time of metamorphosis and then falls further in young frogs. Up to maximum growth 'xanthophyll' storage increases more rapidly than carotene storage. The abrupt fall in 'xanthophyll' level when the rear legs first appeared obviously needs confirmation, but the very small concomitant drop in carotene affords a hint that it is not illusory. The fact that little or no food is eaten during metamorphosis allows the disappearance of carotenoids to be followed. The data given in Fig. 8 and Tables 5 and 6 suggest that the main process does not discriminate sharply between carotene and 'xanthophyll'. The change in the 'xanthophyll'/carotene ratio recorded in Table 4 is not then simply a matter of selective 'utilization' as measured by rate of disappearance. If, during the period of metamorphosis, carotene had been used for the synthesis of vitamin A, the hydrocarbon might have been expected to disappear more quickly than the 'xanthophyll' (mainly lutein in the tadpole). There is no doubt that vitamin A is formed in the tadpole. Natterjack tadpoles each contained about 0·027 μg. in the eyes and newly metamorphosed frogs about 0·1 μg.

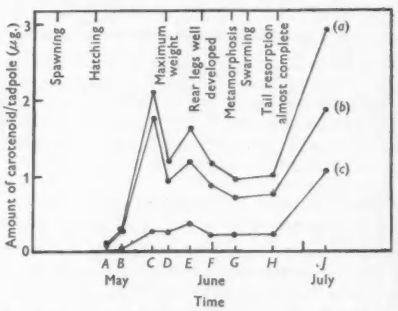


Fig. 8. Carotenoids in the developing tadpole: (*a*) total carotenoid; (*b*) total 'xanthophyll'; (*c*) carotene. The stages *A–H* are specified in Table 4.

It is now clear that the main, if not the sole site of conversion of carotene to vitamin A in mammals is the absorptive portion of the gut, and it seems very probable (although obviously difficult to prove) that the same holds true for the tadpole. It is very unlikely that the 'xanthophyll'/carotene ratio in the kind of vegetation ingested is ever as high as 9; the appearance of this ratio in the early stages of development (*A* and *B*) suggests a selective utilization of carotene in the gut wall. On this hypothesis, as development proceeds and food intake increases, the 'carotenase' enzyme system becomes unable to cope with more than a fraction of the carotene presented to it. The residue will follow the normal pathway of lipid absorption, and the 'xanthophyll'/carotene

ratio will fall until it reaches a figure much nearer to that of the diet. The carnivorous young frogs will continue to convert a proportion of ingested carotene and to absorb carotene and 'xanthophyll' non-selectively.

It is possible that in the future the hatching of frog spawn and the growth of tadpoles in captivity on artificial diets may be of service if either carotene or 'xanthophyll', or both, can be excluded.

Lipochromes and vitamin A in adult frogs

Vitamin A. There does not appear to be any preformed vitamin A in the diet of the frog. The vitamin formed *in vivo* from provitamin is found in the liver (about 85 %) and in the eyes and kidneys in approximately equal amounts (Tables 9 and 11). In each site it occurs mainly as ester. The amount of vitamin A in kidney (about 30 μg./g.) is very much larger than that found in mammalian kidneys.

Carotenoid distribution. Carotene as such is less efficiently absorbed than the hydroxylated carotenoids since the 'xanthophyll'/carotene ratios are approx. 3:1 in the diet, 2:1 in the faeces and, in the body, 4·7:1 for females and 5·7:1 for males (October 1947).

In contrast with the restricted distribution of vitamin A, carotenoids occur in all the organs studied (Table 9). They also appear to occur in nerve tissue and in glands such as the thymus. If the amounts of carotenoids at different sites are expressed in terms of that present in the skin (chosen for comparison because of its relative constancy), the results in Table 7 give an idea of the approximate distribution over the body. In males, the skin, liver and muscle are important; in the female the gonads dominate the situation (Table 7).

Table 7. *Relative amounts of total carotenoid in frog organs*

(These are mostly seasonal averages, without distinction between the sexes.)

Skin*	1
Ovaries and mature eggs	2·65
Liver	0·7
Muscle (total leg)†	0·17
Oviducts	0·12
Immature ovaries	0·1
Stomach	0·05
Testes	0·035
Small intestine, fat bodies, kidneys	0·23–0·28
Oesophagus	0·16
Tongue	0·09
Pancreas, large intestine, lungs, eyes, heart	0·004–0·008
Seminal vesicles, spleen	Traces

* Skin carotenoid is taken as unity, because the absolute amount is large and is less influenced by sex and season than other tissues rich in carotenoids.

† Carotenoids of leg muscle/ventral and side muscle/arm muscle = 36/2/1.

Table 8. *Weights of frog organs expressed as percentage of body weight*

Organ	Minimum	Maximum
Testes	0·32	3·66 (Aug.)
Ovaries	0·925 (Apr.)	36·0 (Mar.)
Oviducts	1·47 (Apr.)	17·5 (Dec.)
Livers (male)	1·4 (Apr.)	3·6 (Nov.–Dec.)
	1·3 (Apr.)	2·3 (Oct.–Dec.)
Fat bodies (male)	Nearly 0 (Apr.)	8 (Aug.–Sept.)
	Nearly 0 (Apr.)	7 (Aug.–Sept.)
Skin	Approx. 10	
Leg muscle	Approx. 15	
Kidneys	0·3–0·5	
Pancreas	0·06–0·13	
Stomach	Approx. 1·0	
Lungs (male)	0·42	
	0·25	
Tongue	0·8	
Eyes	Approx. 1·0	
Heart	Approx. 0·25	

Although the left fat body is larger than the right one, the carotenoid content per unit weight is the same. The adrenals are somewhat richer in carotenoids than the kidneys. In all tissues α-carotene seems to be practically absent and the 'xanthophyll' is nearly all lutein. Very small amounts of other carotenoids were sometimes found, but there was too little for characterization. A few toads (*Bufo vulgaris* and *B. calamita*) have been studied in the course of the present work and no significant differences were observed from experience on frogs.

One of the implications of the distribution data is that the carotenoids are not merely distributed with the fat; thus for females in October 1947 the concentrations of carotenoids in fat varied from about 0·01 mg./g. for fat bodies to 8·4 mg./g. for skin, with liver 2·7 mg./g. and ovaries 1·1 mg./g. (Table 15). The external appearance of frogs and toads owes little or nothing to the lipochromes present in the skin. The outer layers are rich in melanins and in pigments soluble in dilute ammonia.

Lederer & Rathmann (1938) found small proportions of vitamin A_2 in *R. esculenta* liver fat, but in the present work with *R. temporaria* no A_2 was observed, confirming the finding of Gillam (1938).

Ultraviolet absorption spectra of lipid fractions from frog organs

The absorption spectra of liver extracts have already been referred to (Fig. 4). Observations on extracts from other organs are recorded. Selective absorption of undetermined origin in the region 280–300 mμ. was recorded in lipids from the eyes of frogs and toad (Fig. 5) and also rather erratically in frog kidneys. With one exception (November 1946) the testes extracts showed continuous end-absorption over the range 250–400 mμ. The aberrant sample showed an inflexion near 265 mμ.

Extracts from ovaries were more interesting. Material from the mature ovaries of frogs in the prehibernation period exhibited the well-resolved absorption shown in Fig. 9. The characteristic curve

Table 9. *Distribution of fat, carotenoid and vitamin A in the organs of sixty male frogs, divided into three main groups*

Group	Mean body length (cm.)	Mean weight (g.)
A	6·6	22·8
B	5·6	14·2
C	5·1	11·0

In table below means were not measured; a dash (—) means 'not measured'.

Organ	Wt. as % body wt.	Wt. of fat (mg./frog)	Total carotenoid $Q \times 10^2$*	Carotene (μg./frog)	'Xanthophyll' (μg./frog)	Vitamin A (μg./frog)
Fat bodies						
A	0·058	5·6	0·33	0·158	1·40	0
B	0·061	5·4	0·08	—	—	0
C	0·030	2·5	0·02	—	—	0
Pancreas						
A	0·117	—	0·11	—	—	0
B	0·105	—	0·06	—	—	0
C	0·120	—	0·057	—	—	0
Kidneys						
A	0·35	—	0·29	—	—	5·3
B	0·35	—	0·235	—	—	2·4
C	0·35	—	0·18	—	—	1·6
Testes						
A	1·20	4·1	0·33	0·59	0·77	0
B	1·24	2·7	0·22	0·38	0·56	0
C	1·23	1·7	0·17	0·32	0·40	0
Eyes						
A	0·86	—	—	—	—	3·0
B	1·02	—	—	—	—	2·3
C	1·30	—	—	—	—	2·1
Liver						
A	2·31	3·6	4·46	3·4	13·0	39·6
B	2·31	2·9	3·84	3·3	11·5	20·6
C	1·92	2·2	2·32	1·25	7·2	12·1
Tongue						
A	0·63	—	0·11	—	—	0
B	0·74	—	0·082	—	—	0
C	0·72	—	0·078	—	—	0
Stomach						
A	1·09	—	0·48	0·52	1·24	0
B	1·12	—	0·44	0·49	1·16	0
C	1·27	—	0·34	0·35	0·90	0
Skin						
A	11·6	16·2	12·5	7·25	43·6	0
B	11·1	12·6	8·5	5·02	27·8	0
C	11·1	9·7	6·9	2·19	24·4	0
Leg muscle						
A	17·3	18·3	3·42	1·04	10·6	0
B	15·6	10·6	2·04	0·97	6·9	0
C	14·5	6·8	1·54	0·69	5·0	0

* See p. 615 of text.

with maxima at 292·5, 281·5, 271 and 261 mμ. was reproduced in the non-saponifiable fraction. The spectrum is qualitatively indistinguishable from that shown by provitamins D, e.g. 7-dehydrocholesterol and ergosterol. Without using a large amount of material it is not possible to decide which of the several substances known to exhibit this spectrum occurs in the frog ovaries. Applying a correction procedure based on the method of Morton & Stubbs (1946) using reference data obtained on highly purified ergosterol (which is spectroscopically almost identical with 7-dehydrocholesterol) the corrected $E^{1\%}_{1\,cm.}$ 281·5 mμ. value was 0·117, corresponding with a concentration of 1 in 2430 in the ovaries [$E^{1\%}_{1\,cm.}$, (ergosterol) = 285]. A calculation based on the work of Boyd (1938) indicates

that mature ovaries contain about 0·8 % of cholesterol. It thus appears that the ovarian steroids contain approx. 0·5 % of 7-dehydrosteroid.

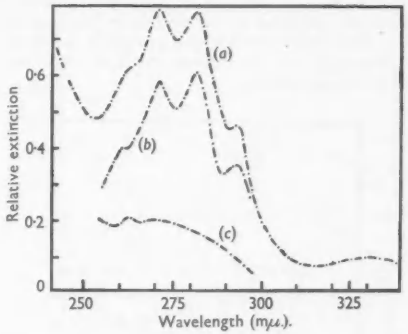


Fig. 9. Ultraviolet absorption spectra of the extract of mature frog ova in ethanol: (*a*) gross absorption; (*b*) corrected curve; (*c*) irrelevant absorption.

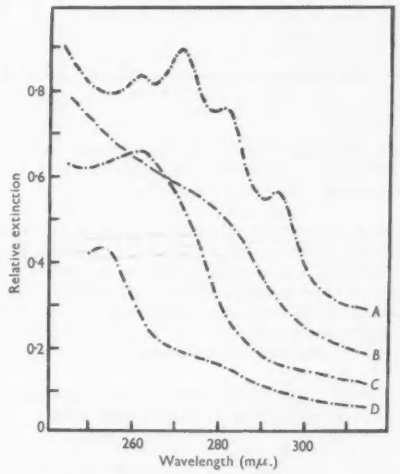


Fig. 10. Ultraviolet absorption spectra of frog ova extracts: *A*, mature ova just prior to oviposition (ethanol); *B*, December ova immature through deprivation (ethanol); *C*, immature ova from frogs which yielded mature ova (curve *A*) (in ethanol); and *D*, immature ova from spring frogs 1947 (*cyclo*hexane).

The characteristic spectrum only appears when the ovaries are mature, but it persists through the period of hibernation and is seen (Fig. 10*A*) just before oviposition occurs. There is some loss of definition and possibly a decrease in intensity, but it is difficult to say how much of the apparent loss is fictitious, since the protein layer and water are accumulated at this time. Curve *C* (Fig. 10) shows the absorption spectrum of an extract of immature ovaries obtained from the same frogs as the ripe ova in March 1948. Curve *D* refers to immature ovaries obtained in the spring of 1947 and *B* to ovaries from frogs which had fasted for a long time (p. 625). The weak selective absorption at 330 mμ. (Fig. 9) is not due to vitamin A or carotenoids.

The data on extracts from other organs show no well-defined spectra, but there are numerous indications of the presence of traces of absorbing constituents. Much larger amounts of material would have to be used in following up these observations.

The occurrence of the ergosterol-like bands in mature ovaries is surprising. It suggests that the hypothesis of a role for 7-dehydrosteroids other than that of a provitamin D is worthy of study.

Seasonal variations in carotenoids and vitamin A

A considerable body of metrical data accumulated during the study of some 600 frogs, the main features of which are summarized in Tables 8 and 10. The quantitative picture which emerges from a study extending over only two seasons is in some respects rather tentative, but it is likely to be broadly correct. In 1946 the liver fat for both sexes showed a 'xanthophyll'/carotene ratio (*X*/*C*) of 1 in the autumn, but a year later *X*/*C* was about 3 (Table 12). The

Table 10. *Size and weight of frogs used at different dates in the present work*

(The average body lengths were computed from the data of March (1937), except those marked with an asterisk.)

Date of dissection	Number		Average body wt. (g.)	Average body length (cm.)
	Males	Females		
14. viii. 46	—	18	27·5	6·9
19. viii. 46	18	—	20·4	6·5
9. ix. 46	20	—	30·5	7·3
11. ix. 46	—	19	25·6	6·9
17. x. 46	18	—	26·8	6·9
21. x. 46	—	18	26·7	6·5
6. xi. 46	20	—	29·3	7·1
12. xi. 46	—	20	34·5	6·9
9. xii. 46	19	—	25·4	6·7
16. xii. 46	—	21	31·1	6·7
1. iv. 47	—	13	29·5	6·6
7. iv. 47	18	—	22·7	6·2
13. iv. 47	—	14	14·5	6·5
12. v. 47	20	—	20·3	6·5
28. v. 47	—	19	16·3	6·3
8. vii. 47	14	—	25·3	7·1
22. vii. 47	—	19	19·9	6·7
3. x. 47	17	—	13·1	5·5*
8. x. 47	—	17	16·8	5·9*
30. x. 47	20	—	22·8	6·6*
30. x. 47	20	—	14·2	5·6*
30. x. 47	20	—	11·0	5·1*

weather remained mild until early December in 1946 and the frog fat bodies were much larger in October and November than a year later. Apart from complications arising from the fact that no two years have the same weather conditions,

the age (size) variable must be distinguished from the variable of an annual cycle in the frog at all ages after puberty.

A group of sixty male frogs, captured near Norwich in October 1947, was divided into three groups. Table 9 summarizes the experimental data. The weights of organs expressed as percentage of body weight are reasonably constant apart from the cases of fat bodies and eyes. No simple expression can be found to cover the lipochrome or vitamin A variations, but both increase with increasing age.

An experiment with a further group of sixty male frogs caught in the spring of 1948 at Hillside showed that two groups of twenty not differing in average size (6·7 cm.) differed little in respect of skin carotenoid, liver carotenoid and liver vitamin A. A third group of the same size showed very much less liver vitamin A and slightly less liver carotenoid. The frogs in the third group, unlike the others, were spent (seminal vesicles empty, and testes shrunken), and, as will be seen, it is plausible to connect that fact with the decrease. Only rather gross signs of seasonal variations, reinforced by data on the average body length of the frogs employed (see Table 10), can therefore be regarded as significant at this stage in the problem.

The variations in carotenoid and vitamin A content of certain organs during the annual cycle are illustrated in Figs. 11–15. The results obtained on the first sixty male frogs in groups *A*, *B*, *C* (p. 620) have been taken into account in the discussion.

Vitamin A

(i) *Liver.* Males and females showed a summer maximum with a fall in early autumn and a rise in late autumn (Fig. 11). There may have been a real decrease prior to

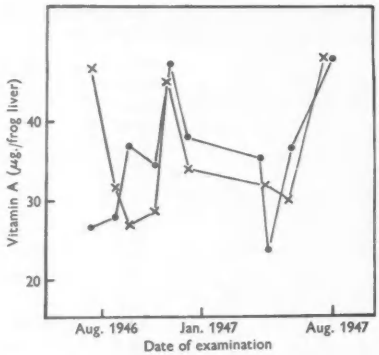


Fig. 11. Seasonal variation in frog liver vitamin A: ×–×–×, male; –·–·–, female.

hibernation, but there was no sign of a drop during winter. Some females shed their eggs in the laboratory without pairing (April 1947) and showed low liver levels afterwards. Similarly, in 1948, the three sets of males already referred to showed respectively 36·1, 36·2 and 20·7 μg./liver, the low value being obtained in the 'spent' animals.

(ii) *Eyes.* Both sexes showed a late autumn maximum and a fall in vitamin A before hibernation. Males but not females appeared to exhibit a winter minimum (Fig. 12).

(iii) *Kidney.* Beyond the fact that kidney storage of vitamin A seems to be maximal in autumn, there is too little evidence for postulating a true annual cycle (Fig. 12). There is little evidence that hibernating frogs deplete their vitamin A reserves, but reproduction causes a heavy drain. The temperature coefficient of the enzyme system responsible for the conversion of provitamin A to vitamin A is probably more responsible for the summer rise in the liver reserve than the copious feeding.

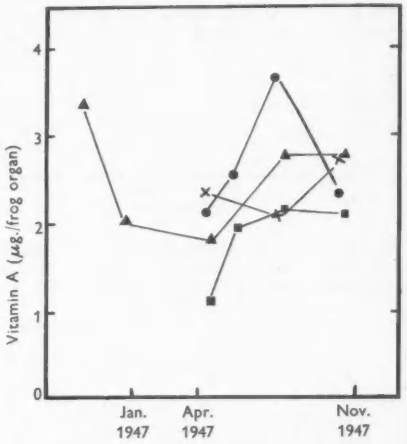


Fig. 12. Seasonal variation in frog eye and frog kidney vitamin A: ×, eye (male); ●, kidney (male); ▲, eye (female); ■, kidney (female).

Table 11 shows for frogs at different seasons the vitamin A contents of the three organs expressed as percentage of the total reserves. The seasonal variations suggest sex differences, and show clearly that the liver is always the main storage organ. So far as the data go, 75–88% of the vitamin A occurs as ester in all sites with 80% as a typical figure. A few discrepant observations were, however, made.

Table 11. *Seasonal variations in the distribution of vitamin A in frogs of both sexes*

Season	Sex	Average body length (cm.)	Vitamin (% of total) Liver	Eyes	Kidneys
Spring	M.	6·2	87·0	6·9	6·1
	F.	6·5	88·4	7·2	4·5
Summer	M.	7·1	88·8	4·1	7·1
	F.	6·7	90·2	5·5	4·3
Autumn	M.	5·5	77·5	11·7	10·9
	F.	6·0	82·7	9·9	7·5

Total carotenoid

(i) *Liver.* No reduction was manifested during hibernation for either sex, in fact there may have been a small rise for males. In both males and females the decrease on mating was followed by a rise to a summer maximum. There was

then a gradual decrease continuing until December with nothing analogous to the autumnal rise in hepatic vitamin A (Fig. 13).

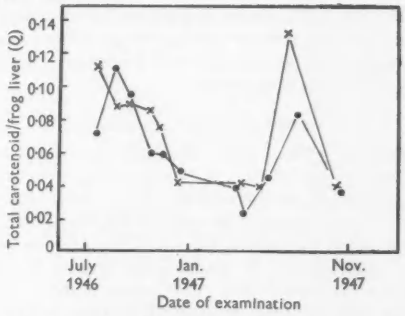


Fig. 13. Seasonal variation in frog liver total carotenoid: ×–×, male; ●–●, female. (*Q*, see p. 615.)

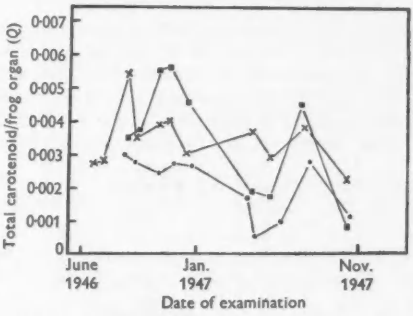


Fig. 14. Seasonal variations in testes and fat body total carotenoid: ×–×, testes; ●–●, female fat body; ■–■, male fat body. (*Q*, see p. 615.)

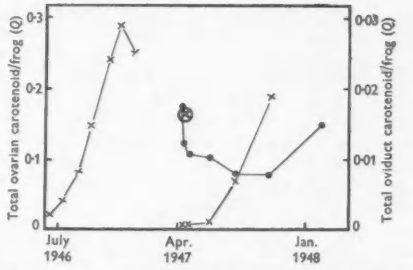


Fig. 15. Seasonal variation in ovarian and oviduct total carotenoid: ×–×, ovaries; ●–●, oviducts. The ringed cross represents the total carotenoid content of mature ova just prior to oviposition. (*Q*, see p. 615.)

(ii) *Sex organs* (Figs. 14 and 15). The gonads and also the fat bodies showed marked seasonal variations in carotenoid content. The testes were very poor in lipochrome after mating and remained so until August when a sharp rise occurred; there was some fluctuation from September to November, but no clear-cut secondary maximum was recorded. Transfer to the testes seems to occur during or just after hibernation. Immature ovaries contain very little carotenoid, but just prior to hibernation a thirtyfold rise had been attained. Whether or not there is a drain on ovarian carotenoids during the winter sleep cannot be stated, as contradictory results were obtained in the 1947 and 1948 seasons, but it could not be large. The oviducts showed a rise in late autumn, but there was no drop in winter (when the ducts are functioning actively). On spawning, the ducts atrophy and there is a sudden decrease to a low level which is maintained through the summer.

Male fat bodies showed a marked rise in November, whereas in females the levels were lower and fairly constant throughout the autumn. Hibernal losses occurred in both sexes and for females especially, marked depletion occurred on spawning, with recovery during summer feeding to lower levels for females than for males.

(iii) *Kidneys, skin and organs of the digestive tract.* No significant seasonal variations in kidney or skin carotenoids could be observed for either sex. Some variations occurred in the digestive tract. Males showed summer and autumn peaks in the stomach, but females only showed the autumn rise. In the small intestine there was a maximum in autumn for both sexes. During winter the intestinal carotenoids were maintained (males) or even rose a little (females).

(iv) *Leg muscle.* A temporary rise appeared to occur in the autumn.

(v) *Tongue, lungs, pancreas, eyes.* There was evidence of an autumn maximum, but the levels were fairly steady on the whole. In the male pancreas an increase is suggested during or immediately after hibernation, but in the female there is a fall on egg-laying. In the male eye the total 'xanthophyll' drops in early summer and then rises, but there is no clear-cut autumn maximum in the female. Carotene was found in traces (<0·05 μg./eye) but 'xanthophyll' preponderates in the eye.

Comment on seasonal variations

The foregoing results are not easy to interpret fully. A tendency for carotenoid content to rise after heavy feeding is quite definite, but some organs respond quickly and others much more slowly. Furthermore, there may be a seasonal change in storage sites. For example, during the early summer-feeding flush, the liver lipochrome increases markedly, yet the muscle content does not rise appreciably; during the autumn feeding this is reversed. However, it is not possible to distinguish between carotenoids carried with fat in lipid transfer and carotenoids mobilized in accordance with the cycle of specific utilization if such exists.

The gonadal cycle in the female results in a larger storage of carotenoids in the developing ovaries than in the whole of the rest of the body. Expulsion of ova is by far the largest cause of carotenoid loss, probably larger even than the total oxidative degradation. The cycle in the testes and oviducts is not responsive to dietary changes, but this is no more than a hint at functional significance. The sex difference in carotenoid storage in the fat bodies recalls the dissimilar gonadal sequence, for spermatogenesis is spread over

no more than six weeks, whereas ovarian development proceeds steadily until the autumn acceleration of growth.

Although muscle and kidney stores fluctuate relatively little it is noticeable that the absolute amount stored in muscle (especially for males) is quite appreciable. Muscle fat and carotenoid is probably mobilized readily. It is interesting that, although frogs feed soon after mating, carotenoid storage does not occur until late May. There is a similar lag in liver glycogen and blood sugar increment (Smith, 1947).

The 'xanthophyll'/carotene ratio (X/C)

The relative amounts of 'xanthophyll' and carotene stored differ markedly in the various organs. For frogs of different sizes the quotient varies as follows: testes 1·2–1·5, liver 3·5–5·8 and stomach 2·4–2·55, skin 5·5–11, muscle 7–10. There is, however, no uniform trend with increasing age in different organs. The data on seasonal changes are summarized in Table 12. It is quite possible that the picture is distorted by some local or other factor connected with the availability of particular kinds of food; in fact marked annual differences have been observed. Some of the grosser effects can, however, be interpreted. When carotene predominates as it appears to do in female livers (August–December 1946), transfer of 'xanthophyll' from liver to gonads is the probable explanation, because the quantitative demand of female gonads for 'xanthophyll' is great. During spermatogenesis (when the total carotenoid is maximal) the X/C ratio for testes is doubled, but the reason is not clear.

Free and combined 'xanthophyll'

Seasonal effects. Table 13 shows the distribution of free 'xanthophyll', and mono- and di-esterified 'xanthophyll' as a percentage of the total. Although high accuracy cannot be claimed for the figures it is obvious that the organs fall into two classes in which the 'xanthophyll' is respectively mainly free and mainly combined. The significance of the data in Table 13 is not clear, because it implies a distribution of esterases for which no explanation can be offered.

Comparison of fat and carotenoid storage

Since lipid is the normal vehicle for carotenoids it is important to know whether the storage and mobilization of lipochrome is correlated with that of fat. Tables 14 and 15 show that it is not. (The ratio fat/carotenoid (C/F) is approximate, since the figures for total carotenoid are obtained by the roughly

Table 12. *Seasonal changes in the 'xanthophyll'/carotene ratio in frog organs*

		1946						1947				
Organs	Sex	July	Aug.	Sept.	Oct.	Nov.	Dec.	Apr.	Apr.	May	July	Oct.
Testes	—	—	2·5 (?)	1·95	1·06	0·87	1·06	0·85	—	0·88	1·92	1·42
Ovaries	—	7·2	6·3	7·2	7·2	6·7	7·4	7·7	11·6	9·6	11·2	8·2
Fat bodies	M.	—	—	7·7	5·7	5·0	4·9	6·7	—	16·0	10·9	7·9
	F.	—	—	—	5·0	4·4	—	—	7·2	5·1	9·2	—
Skin	M.	—	—	—	—	—	—	5·0	—	4·6	5·3	4·8
	F.	—	—	—	—	—	4·1	2·6	4·3	4·1	4·6	2·8
Liver	M.	1·12	0·72	1·97	1·59	1·19	1·46	1·51	—	1·57	1·74	3·0
	F.	1·02	0·51	0·78	0·88	0·71	0·95	0·81	1·58	1·38	1·74	2·8
Kidney	M.	—	—	—	—	—	—	3·6	—	—	5·0	4·5
	F.	—	—	—	—	—	—	2·9	—	2·7	9·3	2·2
Leg muscle	M.	—	—	—	—	—	—	—	—	—	10	7·5
	F.	—	—	—	—	—	—	—	—	—	8·3	8·7
Lungs	M.	—	—	—	—	—	—	3·0	—	—	—	—
	F.	—	—	—	—	—	—	—	—	—	3·9	9·0
Pancreas	M.	—	—	—	—	—	—	6·8	—	—	15·8	7·7
	F.	—	—	—	—	—	—	—	—	—	—	6·2
Stomach	M.	—	—	—	—	2·1	1·7	1·8	—	2·4	—	2·1
	F.	—	—	—	—	—	—	—	—	—	2·4	—
Intestine	M.	—	—	—	—	—	4·1	2·6	—	—	—	4·0
	F.	—	—	—	—	2·6	—	—	—	—	4·2	3·1
Large intestine	M.	—	—	—	—	—	—	1·9	—	—	—	0·9
	F.	—	—	—	—	—	—	—	—	—	2·3	1·0
Tongue	M.	—	—	—	—	—	—	—	—	—	3·8	1·1
	F.	—	—	—	—	—	—	—	—	—	1·8	2·2
Oesophagus	M.	—	—	—	—	—	—	—	—	—	—	2·4
	F.	—	—	—	—	—	—	—	—	—	4·9	—
Oviducts*		—	—	—	—	—	—	0·83	0·67	0·76	0·72	1·0

* Oviducts alone fail to show 'xanthophyll' predominating at any season of the year. X/C ratio was 0·88 in Jan. 1948.

Table 13. *Seasonal distribution of free 'xanthophyll' and xanthophyll mono- and di-esters in frog organs*

Organ	Season	Sex	Percentage of total 'xanthophyll' as Di-ester	Mono-ester	Free
Skin	Summer	F.	68	15	27
Skin	Summer	M.	61	19	20
Skin	Autumn	M.	68	15	17
Skin	Spring	M.	74	13	13
Ovaries	Summer	—	52	33	15
Ovaries	Autumn	—	60	27	13
Ova	Spring	—	58	20	22
Liver	Autumn	M.	9	18	73
Muscle	Autumn	M.	12	8	80
Muscle	Autumn	F.	8	8	84
Muscle	Spring	F.	10	9	81
Oviduct	Autumn	—	11	22	67
Testes	Winter	—	7	23	70
Fat bodies	Winter	M.	11	34	55
Small intestine	Winter	M.	6	13	81

Table 14. *Total carotenoids and total fat of organs of male frogs of different sizes*

(Mean body lengths: Group *A*, 6·6; *B*, 5·6; *C*, 5·1 cm. Total carotenoid calculated on basis of mean $E^{1\%}_{1\,cm.}$ 450 mμ. 2500.)

Group ...	*A*			*B*			*C*		
	Fat (mg.)	Carotenoid (μg.)	*C/F* (mg./g.)	Fat (mg.)	Carotenoid (μg.)	*C/F* (mg./g.)	Fat (mg.)	Carotenoid (μg.)	*C/F* (mg./g.)
Testes	4·1	1·3	0·3	2·7	0·87	0·32	1·7	0·68	0·4
Liver	3·6	18	5·0	2·9	15·4	5·3	2·2	9·3	4·2
Skin	16·2	50	3·1	12·6	34	2·7	9·9	28	2·9
Muscle	18·3	13·7	0·75	10·6	8·2	0·77	6·8	6·2	0·9

Table 15. *Seasonal variations* (1947) *in ratio of total carotenoid/fat in frog organs*

(Ratios expressed as mg. total carotenoid/g. fat.)

Organ	Sex	April	May	July	October
Testes	—	—	0·63	0·44	0·42
Ovaries	—	3·4	1·3	1·12	0·68
Fat bodies	M.	—	0·49	0·01	0·09
	F.	—	0·02	0·01	0·12
Kidneys	F.	—	0·66	0·3	1·5
	M.	—	0·28	0·47	1·4
Liver	F.	3·3	3·6	1·6	3·6
	M.	3·2	1·6	2·7	1·1
Leg muscle	M.	—	—	—	1·8
	F.	—	—	0·34	1·2
Skin	M.	2·8	3·5	3·1	3·2
	F.	3·4	2·3	8·4	4·0
Oviducts		0·3	0·3	0·2	0·4

correct assumption of an average $E^{1\%}_{1\,cm.}$ 450 mμ. of 2500 for the constituent pigments.) Variations with size are obviously less important than inter-organ variations. Seasonal variations (Table 15) are striking, but again there is no correlation between fat transfer (or metabolism) and the fate of carotenoids.

Vitamin A fed to frogs

One male and one female were each given 1 drop (approx. 3500 i.u.) of an ester concentrate daily for a month, with no other food. Much of the vitamin was excreted, but the liver level rose threefold. Some vitamin appeared in the extracts from lungs, fat bodies and testes, but not in the skin or the ovaries. The experiment indicates poor assimilation, but this may have been due to the fact that the animals (like all frogs in captivity) refused to eat spontaneously. The absence of vitamin A from the ovaries is consistent with the observation that it is not detectable in newly spawned eggs.

Fasting frogs

The ability to do without food for long periods is a well-known characteristic of frogs. In an experiment in which twenty-five female frogs were kept without food from 18 July to 9 December 1947, the fat bodies had been exhausted and body weights had declined by some 35%. Ovarian development was greatly retarded. The decreases in weight of the viscera during inanition were in general agreement with previous observations (Ott, 1924). Most of the intestines contained faeces of liquid consistency with a 'xanthophyll'/carotene ratio of 4/1 as compared with the normal faecal value of 2. The vitamin A level of the liver had not fallen very much, but the eyes and the kidneys had been depleted; 97% of the total vitamin was found in the liver.

The results recorded in Tables 16 and 17 need little discussion. Perhaps the major point is that despite the great changes incidental to prolonged fasting the overall *X/C* quotient for the whole frog rises to 4·6 exactly as in normal animals (3·6–4·6) over the same period of the year. This seems to eliminate dietary influence and supports the idea of selective utilization of different carotenoids.

Table 16. *Fat, carotenoids and vitamin A in female frogs kept in captivity for* 25 *weeks*

(Average decrease in body weight 35%. Means not measured.)

Organ	Average wt. of organ as percentage of body wt.*	Fat (mg./frog)	Vitamin A (μg./frog)	Carotenoid (μg./frog)	Xanthophyll / Carotene
Liver	0·99	1·29	24·7	7·4	1·97
Eye	1·34	0·72	0·62	—	—
Kidney	0·23	1·21	0·074	0·5	2·09
Skin	9·01	3·39	—	19·2	2·71
Ovaries	5·26	17·0	—	22·1	10·2
Immature ovaries	1·19	2·17	—	8·7	19·0
Oviducts	8·30	5·19	—	1·3	0·78
Immature oviducts	0·94	—	—	0·3	3·37
Tongue	0·83	—	—	0·15	4·92
Pancreas	0·025	—	—	0·15	11·0
Lungs	0·274	—	—	0·13	—
Leg muscle	9·47	3·3	—	4·8	7·25
Oesophagus	0·31	—	—	0·12	—
Stomach	0·75	—	—	0·4	1·78
Intestine	0·43	—	—	0·2	6·37
Large intestine	0·19	—	—	0·05	—

* Compare Table 9 column 2.

Table 17. *Distribution of 'xanthophyll' among the free form, mono- and di-esters in the organs of fasted female frogs*

Organ	Percentage of total 'xanthophyll' existing as: Di-ester	Mono-ester	Free form
Liver	8	18	74
Leg muscle	4	11	85
Skin	69	19	12
Oviducts	25	15	60
Ovaries	37	47	16
Immature ovaries	26	60	14

GENERAL COMMENTS

The present study perhaps makes timely a general comment on the place of distribution studies in the growth of ideas on the biological significance of particular compounds. It is a very uncertain place; the pattern of distribution is a resultant of superimposed patterns, of intake, assimilation, storage and utilization, of precursors and metabolites, and of enzyme capacities and 'detoxication' processes. Sometimes distribution studies seem merely to add new difficulties, but at other times they suggest fruitful lines of attack. A few examples may be given:

Vitamin A. The distribution data are consistent with storage in the liver and with localized function in the eyes, but the apparent absence of vitamin A from normal nerve tissue, bone and mucosa is surprising in the light of the pathology of avitaminosis A. Comparative biochemistry adds to the complexity, e.g. the basking shark is found to store no vitamin A in its liver and the tunny fish to store enormous amounts.

Cholesterol. The concentration of cholesterol in the adrenals pointed to functions, the nature of which is clarified by the effects of stress or injections of adrenocorticotrophic hormone. On the other hand, the broad picture of cholesterol distribution merely emphasizes the inadequacy of existing knowledge.

Provitamin D. The occurrence of 7-dehydrocholesterol in skin fat is now seen to be significant, but its presence in ripe frog ovaries, in intestinal mucosa and in the sterols from worms is no easier to understand than the high concentration of ergosterol in yeast.

As was implied at the outset, this investigation was prompted by the idea that carotenoids may have functional significance in at least some animals. The advantage of the frog is that carotenoids are present at all stages in the life cycle; its disadvantage is that possible clues may be masked by the casual ecological variables and by shifting enzyme capacities. In the present state of carotenoid biochemistry the need is to find a bodily site or a developmental phase marked out by the distribution as possessing special interest.

Research in the field is halted by the lack of serviceable hypotheses. The present work suggests that: (*a*) beneath much that may be casual there are strong suggestions of significance; (*b*) in both males and females, the sexual cycle exhibits patterns of storage, transfer and disappearance of carotene and xanthophyll, consistent with independent and significant roles; (*c*) the whole method of approach is in essence exploratory and its aim is to provoke the experiments which turn surmises into certainties.

SUMMARY

1. The occurrence of carotenoids and vitamin A in frogs (*Rana temporaria*) and tadpoles has been studied and new metrical data have been obtained on size and weight.

2. Analytical methods for the accurate and speedy determination of carotenoids and vitamin A have been described and tested.

3. Whole tadpoles were studied at different stages of development and the changes in total 'xanthophyll' and carotene content recorded. The presence of chlorophyll is ascribed to green food undergoing digestion. The 'xanthophyll'/carotene quotient declines from about 9 to about 3 as development proceeds. Vitamin A was detectable in tadpole eyes.

4. The weights of frog organs expressed as percentage of body weight are recorded and maximum and minimum values given when variations are considerable. Much of the carotenoid actually ingested is contained in undigested food present in the alimentary tracts of insects or other prey. The frog does not ingest preformed vitamin A but converts carotene to the vitamin. About 85 % of the total vitamin occurs in the liver (mainly as ester); the remainder is normally about equally divided between kidneys and eyes. Frog kidneys contain a higher proportion of vitamin A than do mammalian kidneys. Most of the vitamin A in the eye is ester, stored in the choroid. Carotenoids are dispersed over most organs but quite unevenly. The deposition of carotenoids in the ovaries is so large that a marked difference results between the sexes in carotenoid storage and utilization.

5. Mature ovaries contain in relatively high concentration a substance showing the four absorption maxima characteristic of 7-dehydrocholesterol and ergosterol.

6. Data have been obtained on the seasonal variation in different organs of some or all of the following: carotene, 'xanthophyll' (free, mono-ester, di-ester), vitamin A (free and esterified) and total fat. The results on normal and fasting frogs reveal: (*a*) the retention of liver reserves in hibernation and inanition; (*b*) well-defined seasonal variations in the carotenoid storage of different organs; (*c*) some indication of the continuance of carotenoid metabolism during hibernation; (*d*) the overwhelming quantitative importance of the ovarian requirements; (*e*) a drain on vitamin A reserves during reproduction; (*f*) the distinct patterns of storage and utilization of vitamin A, carotenes, 'xanthophylls' and fat in the frog.

We are indebted to the Medical Research Council and the Ministry of Food for financial assistance. One of us (D.G.R.) held an Evans, Lescher and Webb Studentship and later a Johnston Fellowship in the University of Liverpool.

REFERENCES

Ackermann, J. (1938). *Bull. int. Acad. Cracovie*, B. II, 241.
Bartz, J. P. & Schmitt, F. C. (1936). *Amer. J. Physiol.* **117**, 280.
Boyd, E. M. (1938). *J. Physiol.* **91**, 394.
Brunner, O. & Stein, R. (1935). *Biochem. Z.* **282**, 47.
Comar, C. L. & Zscheile, F. P. (1942). *Plant Physiol.* **17**, 198.
Dietel, F. G. (1933). *Klin. Wschr.* **12**, 601.
Eekelen, M. van (1934). *Acta Brev. neerl. Physiol.* **4**, 65.
Gillam, A. E. (1938). *Biochem. J.* **32**, 1496.
Glover, J., Goodwin, T. W. & Morton, R. A. (1947). *Biochem. J.* **41**, 97.
Harris, D. G. & Zscheile, F. P. (1943). *Bot. Gaz.* **104**, 515.
Lederer, E. & Rathmann, F. H. (1938). *Biochem. J.* **32**, 1252.
Lönnberg, E. (1929). *Ark. Zool.* **21**, B (3).
Manunta, C. (1934). *Atti Soc. Nat. Mat. Modena*, **65**, 71.
March, F. (1937). *Proc. zool. Soc. Lond.* **107**, 415.
Morton, R. A. & Stubbs, A. L. (1946). *Analyst*, **71**, 348.
Ott, M. D. (1924). *Amer. J. Anat.* **33**, 17.
Pepkowitz, L. P. (1944). *J. biol. Chem.* **155**, 219.
Rand, C. (1935). *Biochem. Z.* **281**, 200.
Smith, C. L. (1947). Private communication.
Wald, G. (1935). *J. gen. Physiol.* **19**, 781.
Zechmeister, L. & Tuzson, P. (1936). *Hoppe-Seyl. Z.* **238**, 197.
Zscheile, F. P., White, J. W. Jr., Beadle, B. W. & Roach, J. R. (1942). *Plant Physiol.* **17**, 331.

A Histological Method for the Biological Estimation of Vitamin A

By W. H. K. COETZEE,*
Department of Physiology and Pharmacology, University of Cape Town

(*Received* 19 *April* 1949)

There are considerable differences between physico-chemical and biological results of vitamin A estimations, owing to the fact that the 'analyst determines the proportion of a substance present, whilst the bio-assayist determines an effect' (Morton, 1942). The biological vitamin A value of food or pharmaceutical products is the sum of the values of all substances present having vitamin A activity, such as preformed vitamin A, either as the free alcohol or esters, and various carotenoid pigments. The response of the experimental animal to a given dose of test substance is dependent upon the absorption and utilization of vitamin A and its precursors, and is influenced by the digestibility of the food and the absence or presence of antioxidants. Physicochemical estimations of the vitamin A content of a product, on the other hand, depend in the first instance on determinations of intensity of absorption in the region 325–328 mμ. where vitamin A shows its maximal light absorption. Allowance may have to be made for irrelevant absorption.

To convert extinction coefficients, as determined by physico-chemical means, to biological units, a conversion factor has been adopted. This factor, however, is chiefly based on the results of the curative 'growth' method (Coward, 1947) which, although not specific for vitamin A, is more accurate than methods based on examination of the vagina or the eye (xerophthalmia). The results of various doses of vitamin A upon pathological changes in the central nervous system of the rat led Irving & Richards (1940) to suggest that such changes might form the basis of a prophylactic assay of vitamin A. At weaning, rats were placed on a vitamin A-deficient diet and graded doses of the vitamin were administered. When after 7 weeks the rats were killed, degeneration was found in those receiving 1 i.u. or less daily, but not in those receiving 1·5 i.u. or more, and the demarcation was extremely sharp.

The work presented here was undertaken primarily to determine the suitability of the method suggested by Irving & Richards as a prophylactic assay. Arising from this, the accuracy of the present-day values of the conversion factor was also investigated.

* Present address: Western Province Fruit Research Station, Stellenbosch, C.P., South Africa.

Degeneration in the central nervous system due to vitamin A deficiency

Degeneration of the central nervous system in young swine, as a specific symptom of vitamin A deficiency, was described as early as 1914 by Hart & McCollum (1914; cf. Hart, Miller & McCollum, 1916). Their ration contained a high proportion of cereals, and it was believed that cereal toxicity was the cause of nervous degeneration. An unknown factor (vitamin A) 'associated with butter fat and certain other fats and found . . . in considerably higher concentration in the leaves . . . of alfalfa' had a protective action against nervous lesions.

Mellanby (1926) reported severe degeneration of the nervous system in puppies placed on high-cereal diets, and stressed the toxicity of cereals in the absence of fat-soluble vitamins. He suggested the name 'toxamin' for the harmful substance and ascribed its toxicity to interference with calcification of bones. His belief in the toxicity of cereals as the cause of degeneration was held for many years (Mellanby, 1930, 1931, 1934). Subsequent workers have not, however, supported it. Hughes, Lienhardt & Aubel (1929), who described nerve degeneration in pigs resulting from avitaminosis A, stated 'In no case, so far as we are aware, has anyone reported the finding of pathological changes in the nervous system accompanying avitaminosis A.' Zimmerman (1933) produced lesions in the nervous system with a diet containing no cereals. Finally, Irving & Richards (1938) replaced the cereal component of the diet by a mixture of tapioca starch and pure glucose (2:1) and proved that the belief in the toxicity of cereals was not justified.

Age is an essential factor in the production of nerve lesions in vitamin A deficiency. The main factors are the amount of vitamin A stored in the animal body at the beginning of the experiment and the effect of rapid growth in the early stages. This has been found by Suzman, Muller & Ungley (1932), Eveleth & Biester (1937), Wolbach & Howe (1925) and Wolbach & Bessey (1940, 1941).

That loss of weight alone does not account for paralysis or degeneration in the cord, was shown by several workers (Aberle, 1934; Wolbach & Bessey, 1940, 1942). Rats received a diet adequate in

vitamin A but restricted in amount, so that their weights paralleled those of litter mates on a vitamin A-deficient diet. All the rats died after approximately the same experimental period, but, in contrast with the controls, those receiving vitamin A or carotene showed no paralysis or degeneration of the cord. It was also demonstrated that neither tocopherol (Wolbach & Bessey, 1942) nor unsaturated fatty acids (Zimmerman, 1933; Zimmerman & Cowgill, 1936) affects the development of nervous lesions.

The general picture of vitamin A-deficiency degeneration in the central nervous system of the rat has been described by Irving & Richards (1937, 1938). Maximum degeneration was usually found in the funiculus praedorsalis at the level of, or just below, the pyramidal decussation. The degeneration gradually became less, and more diffusely spread, at lower levels in the cervical cord. Microscopical examination of a cross-section revealed the site of maximal degeneration in the anterior columns. As the severity of the lesions increased, the posterior columns and especially the column of Burbach, and the spino-cerebellar tracts, gradually became more affected, though the degeneration never reached the same intensity as in the funiculus praedorsalis. Irving & Richards (1937) stated that the 'uniformity with which the condition described occurs in every one of the rats examined is in striking contrast with the variability in pathological condition, and in the occurrence of epithelial metaplasia found in animals in advanced avitaminosis A. The fact that this degeneration in the medulla occurs with such uniformity before the weight curve is affected to any extent, suggests that it is one of the fundamental lesions of vitamin A deficiency.'

The causation of nerve degeneration in vitamin A deficiency. No worker has so far produced a theory which satisfactorily accounts for this. That vitamin A plays an important role in calcification of bones was pointed out by Copp & Greenberg (1945). They found that fracture callus was much smaller in the deficient rat than in the normal animal and the calcification was less active. Mellanby (1938, 1941, 1947) postulated that vitamin A deficiency upset the regulation of skeletal growth by means of interference with the activity of the osteoblasts and osteoclasts. On a surface where osteoclasts were normally active, their activity could be suppressed and that of the osteoblasts might predominate and vice versa. The resulting bony overgrowth caused pressure on the central nervous system and peripheral nerves by squeezing and stretching and resulted in degeneration due to mechanical injuries.

Wolbach & Bessey (1940, 1941, 1942) and Moore & Sykes (1941) agreed with Mellanby that degeneration was caused by mechanical injuries due to pressure. They, however, ascribed this to causes other than bone growth. The bones ceased to grow with the establishment of vitamin A deficiency, which caused a disproportionate growth of the central nervous system in relation to its bony enclosure. Attention was also drawn to the possibility that vitamin A deficiency accelerated the growth of the central nervous system (Wolbach & Bessey, 1940).

Another factor that needs to be considered here is the increase in spinal fluid pressure that accompanies vitamin A deficiency, as was found by Moore & Sykes (1940, 1941) in bovines. It was suggested by these workers that inco-ordination and nervous lesions might be related to increases in the cerebrospinal fluid pressure. This was due to an increased volume of the cranial contents, caused by either a relative overgrowth as indicated by Wolbach & Bessey, or to bone overgrowth as postulated by Mellanby.

These data indicate that, whatever the mechanism of vitamin A-deficiency degneration may be, it is still obscure. As far as the hypothesis of cessation of bone growth is concerned, the work of Orr & Richards (1934) and of Irving (1948) disproves it. Bone overgrowth and increased intracranial pressure have been definitely demonstrated. If, however, degeneration was due to any kind of pressure effect, an even distribution of demyelinated fibres would be expected in a cross-section of the cord and not the well-defined degeneration that has been found always to occur in the same tracts.

EXPERIMENTAL

Diet. The composition of the vitamin A-free diet was as follows: Casein, 17; potato starch, 47; arachis oil, 14·1; brewer's yeast, 17·2; salt mixture (McCollum 185), 4·7%.

Owing to post-war restrictions it was impossible to obtain cornstarch and so potato starch was used instead. The arachis oil was obtained in one-gallon tins and was of a good quality. The colour varied from a light straw colour to light brown and no vitamin A or carotenoids could be detected in it by the $SbCl_3$ test. Before using a new tin it was fortified with calciferol so as to obtain approximately 100 i.u. vitamin D/kg. of mixed diet. It contained enough tocopherol to give maximum vitamin A protection. The casein was extracted with 96% ethanol for about 7–8 hr. before using, care being taken not to overheat the casein during this treatment, in order to avoid diarrhoea in the experimental animals. It is said that rats require no ascorbic acid, but to be on the safe side one ground tablet, equal to 50 mg. vitamin C, was included in every 2 kg. of diet. The vitamin A-free food mixture (without the arachis oil) was mixed in quantities of 3 kg. at a time. Each day the necessary amount of this dry food mixture was weighed out and the corresponding amount of KI solution (0·64 g./l.) added, namely 0·5 ml. for every 70 g. of the former. The oil was rubbed in and afterwards enough water was added to make a stiff paste, which was crumbled up and put into the food pots.

Rats. Young rats of the Wistar Institute strain were employed. Litters of more than nine were reduced to that number at birth. The whole litter, including the mother, was

placed on the vitamin A-free diet 5 days before weaning, which was done at 22 days of age. This procedure had been adopted by Irving & Richards (1938), who found that it produced more consistent results, and that the test animals so treated showed signs of deficiency 7 or more days before those not put on the deficient diet before weaning.

As a general rule, the litters contained more females than males. To save time and material, two different preparations were tested simultaneously against the same standard and with three groups for every oil solution; each assay consisted of nine dosage groups. With a limited number of litters, it was not always possible to start such an experiment with equal numbers of males and females (5, 5) in each group. The distribution was, therefore, always carried out with the main object of starting an experiment with ten rats in each group which consisted as far as possible of five males and five females and to minimize the influence of litter variations.

Oils tested. Biological experiments were carried out on samples of the following, the spectroscopic characteristics (Table 8) of which were also determined first:

(1) Vaalhaai (*Galeorhinus capensis*) liver oil.
(2) Vitamin A acetate in arachis oil.
(3) Vitamin A distillate in arachis oil.
(4) Snoek (*Thyrsites atun*) liver oil.
(5) Hake (*Merluccius capensis*) liver oil.
(6) Tuna (*Germo albacora*) liver oil.
(7) Dogfish (*Squalus* species) liver oil.
(8) Stonebass (*Polyprion americanus*) liver oil.

Except for the vitamin A acetate and distillate the rest were natural fish-liver oils, where the vitamin A exists chiefly in the form of esters. Determinations of extinction coefficients were made with the Beckman spectrophotometer using *cyclo*hexane as solvent and only in the case of the dogfish-liver oil was the estimation carried out on the unsaponifiable fraction. The vitamin A acetate and the distillate were dissolved in arachis oil to an E value of about 7 and the spectrophotometric estimations were carried out on the solutions.

Conduct of the experiment. To obtain the final dosing solutions, the oils were diluted in steps with arachis oil to a potency of about 60 i.u./g. as calculated from the $E^{1\%}_{1\,cm.}$ values using 1600 as conversion factor. Fresh dosing solutions were made up every 2 weeks from a solution of intermediate strength. All the oils were stored in the refrigerator in glass-stoppered bottles. International β-carotene standard and vitamin A acetate were used as biological standards and each experiment was a comparison between standard and test oil responses which were obtained simultaneously. The solution of crystalline vitamin A acetate was made up in pure arachis oil to an $E^{1\%}_{1\,cm.}$ value of 6·85. This was used as a standard of reference in a further diluted form after standardization against the International β-carotene standard. Thus the β-carotene preparation was accepted as the primary standard throughout. The U.S.P. vitamin A acetate standard was not used. The acetate referred to was a crystalline preparation obtained commercially.

Dosing started at the beginning of the experiment and vitamin A was administered orally every 3 days by means of a tuberculin syringe with a micrometer-screw attachment, which released a standard drop of 0·01 g. (= 0·6 i.u. vitamin A) per turn. Preliminary experiments showed that satisfactory results were obtained when vitamin A was administered every third day. The different dosage groups received, therefore, 1, 2 and 4 turns per rat every third day, which was equal to approximately 0·2, 0·4 and 0·8 i.u. of this vitamin per day. These three levels were selected since it was expected that the dosage-response curve would be a straight line when the response was plotted against the log. of the dose. Owing to the possibility that the time of dosing might have an influence on the absorption and utilization of vitamin A and β-carotene, dosing was always carried out before feeding. The test period lasted for 35–42 days and the weights and eye conditions were taken as criteria as to when to stop the experiment, when the rats were killed with coal gas.

Histological examination of the central nervous system. In the demonstration of degeneration in the nervous system, most workers prefer to use the Marchi method, whilst Kultschitzky's stain and polarized light (Lee & Sure, 1937; Setterfield & Sutton, 1935) are less often applied. In the present experiments the Marchi technique was employed. Irving & Richards (1938) showed that, if proper precautions were taken, this method gave reliable and reproducible results; the method employed followed in the main that adopted by them. Though the Marchi technique is usually considered rather slow, a procedure was evolved whereby it was possible to embed eighty medullas in 1 day. A résumé of the method is therefore given for the benefit of other workers who may use this method of assay.

Rats were killed with coal gas in groups of about ten each. Part of the skin and the muscles on the back were removed to expose the skull and upper cervical vertebrae but without removing the ears, which had distinctive markings. The cranium and spinal canal were opened with great care to avoid injuring the medulla. Having finished that operation, the skull with the central nervous system inside was cut off from the rest of the body. Owing to the tension of the ligaments the cervical vertebrae were then pulled at an angle to the skull. To avoid fixing the medulla in that right-angled position, the axis of the skull was alined with that of the spinal cord by inserting a sharpened match stick through the trachea into the mouth.

The skulls of the rats belonging to different dosage groups were placed in bottles containing 2·5% (w/v) aqueous $K_2Cr_2O_7$ solutions with corresponding labels. After 5 days the dissection was completed by removing the lower medulla and upper cervical cord (subsequently referred to as the medulla), which was transferred to the same strength of $K_2Cr_2O_7$ solution in a cork-stoppered, flat-bottomed specimen tube, where it remained for a further 5 days. The nervous tissue was very soft at that stage, and it was necessary to handle it with the utmost care to avoid squashing it between the fingers.

The 2·5% $K_2Cr_2O_7$ solution was next poured out and about 3 ml. of a mixture consisting of one part of a 1% (w/v) osmic acid solution in two parts of a 5·3% (w/v) solution of $K_2Cr_2O_7$, thoroughly mixed in a glass-stoppered measuring cylinder, was put into the tube containing the specimen. That solution was changed for a similar one after 5 days for the same period. The tissues were then washed, dehydrated in ethanol, cleared in $CHCl_3$ and embedded in paraffin.

Sections (10 μ) were cut serially starting from the cord end. Two out of every ten were taken and arranged in sequence on the microscope slide in two parallel rows of ten each. As a general rule, Marchi-stained nervous tissue is very brittle, but the use of the following precautions seemed to improve the method, and sections without any cracks

were obtained. Care was taken to perform the transference of the medullas from the $CHCl_3$ to the wax baths as quickly as possible, lest the tissues should dry and cracks appear by shrinkage. A vacuum oven was used for the embedding and it was found important to decrease the pressure very gradually as a rapid decrease also resulted in cracks.

Criterion of response. Irving & Richards (1938, 1940) indicated the degree of severity of the nervous lesions by numbers according to the following scheme: stage 1 –, a very few fibres degenerated; stage 1, a few scattered fibres degenerated; stage 2, well-marked degeneration; stage 3, very marked degeneration; stage 4, heavy degeneration.

They added that the degree of degeneration represented by the numbers of 1 and 1 – was of no significance, since an exactly similar effect was observed in positive controls and stock rats, and was an artefact of the Marchi technique. This experience was not confirmed in the present work. No degenerated fibres could be detected under the low power of the microscope in stock rats and positive controls, and the slightest degeneration found in the experimental rats was, therefore, taken as an indication of the first vitamin A-deficiency symptom.

The classification used by Irving & Richards was found to be unsuitable for assay purposes since statistical methods could not be applied. It was found, however, that with a little experience the degeneration could be divided into six different stages, thereby increasing the accuracy of the estimation.

With the object of obtaining a positive curve of response it was decided to use the degree of protection against degeneration as the criterion of response. The value 0 was assigned to a section showing maximal degeneration to indicate that the degree of protection against degeneration was 0 in this case. A section with no degenerated fibres received the value 5 indicating full protection. (See Figs. 3–8, Pls. 6 and 7.)

For the classification of the slides, two microscopes with mechanical stages were used. Slides belonging to the same assay were first classified roughly with one microscope in different groups according to their 'stage of protection'. A standard slide, say of stage 4, was next put under one microscope and slides which were expected to show approximately the same protection were examined individually under the other microscope. By finding first the section in

Table 1. *Estimated degree of protection against myelin degeneration in male rats given by different doses of vitamin A*

(Each numbered experiment gives the result of assays carried out simultaneously. The International Standard was the β-carotene standard preparation, as issued by the National Institute for Medical Research, which was used after further dilution with pure arachis oil to a potency of 60 i.u./g. The vitamin A acetate solution ($E^{1\%}_{1\,cm.}$ 6·85, see p. 630) was prepared from crystalline material. The letters (*a*), (*b*) and (*c*) below and in the following tables refer to duplicate or triplicate assays carried out on the same preparation. The potency of liver oils was roughly assessed by the gross $E^{1\%}_{1\,cm.}$ value $\times 1600$. D.P. = degree of protection.)

		Dose					
		0·2 i.u./day		0·4 i.u./day		0·8 i.u./day	
Exp. no.	Preparation	No. males	D.P.	No. males	D.P.	No. males	D.P.
1	Internat. Std. (*a*)	3	1·5	4	5·0	4	10·0
	Vaalhaai liver oil (*a*)	3	2·0	3	4·5	4	9·5
2	Internat. Std. (*b*)	2	1·5	4	2·0	4	4·5
	Vaalhaai liver oil (*b*)	2	0·5	4	2·5	4	6·5
3	Vit. A acetate (*a*)	3	6·5	4	11·5	4	14·0
	Internat. Std. (*a*)	3	7·0	4	9·5	4	16·0
	Vit. A distillate (*a*)	3	8·5	4	9·0	4	16·5
4	Vit. A acetate (*b*)	5	10·0	5	10·5	5	16·5
	Internat. Std. (*b*)	5	9·5	5	9·5	5	15·0
	Vit. A distillate (*b*)	5	12·5	5	14·0	4	15·5
5	Snoek liver oil (*a*)	4	3·5	4	7·5	5	13·0
	Vit. A acetate Std. (*a*)	4	1·5	5	9·5	4	10·0
	Hake liver oil (*a*)	3	1·5	4	7·5	5	16·5
6	Snoek liver oil (*b*)	6	4·0	6	10·0	5	10·5
	Vit. A acetate Std. (*b*)	6	5·5	5	6·0	5	18·5
	Hake liver oil (*b*)	6	7·0	6	10·0	5	15·0
7	Tuna liver oil (*a*)	5	6·0	5	12·0	5	19·0
	Internat. Std. (*a*)	5	6·0	5	11·5	5	19·0
	Dogfish liver oil (*a*)	4	4·5	5	8·5	5	19·0
8	Tuna liver oil (*b*)	5	2·5	5	10·5	5	14·5
	Internat. Std. (*b*)	5	7·0	5	8·5	5	15·5
	Dogfish liver oil (*b*)	5	4·0	4	4·5	5	11·0
9	Stonebass liver oil (*a*)	5	1·5	4	2·0	5	13·0
	Vit. A acetate Std.	6	4·5	6	5·5	6	18·5
	Stonebass liver oil (*b*)	4	2·5	5	8·5	4	8·0
	Vaalhaai liver oil (*c*)	4	2·0	4	5·0	5	12·5
	Totals	111	123·0	120	205·0	121	357·5
	Weighted means		**1·108**		**1·708**		**2·954**

the two rows revealing the maximal degeneration and, secondly, by comparing the number and size of the black areas in this section with those of the standard under the first microscope, it was easy to recognize the actual stage of protection. If the black spots showed a similar intensity to that of the standard, that specific slide was taken as stage 4, while a little more degeneration denoted stage 3·5 and a little less stage 4·5. This procedure was repeated for every group. After a little experience had been gained, it was found that the classification of the same slides undertaken a few weeks later showed no significant difference.

RESULTS

Difference in response of males and females. Coward (1932) described the difference in response to vitamin A dosage of male and female rats. She found that males gave results with a greater standard deviation than that given by female rats. However, the steeper slope of the curve of response of the former caused them to give results of greater accuracy than those obtained with female rats. Brenner, Brookes & Roberts (1942) observed a significant sex difference in the liver storage of vitamin A in rats. 'The females stored and retained more vitamin A in the liver than the males, while the males had higher blood values.' Using the fluorescence technique to demonstrate vitamin A in the liver, Popper & Brenner (1942) came to the conclusion that 'During depletion the livers of male rats

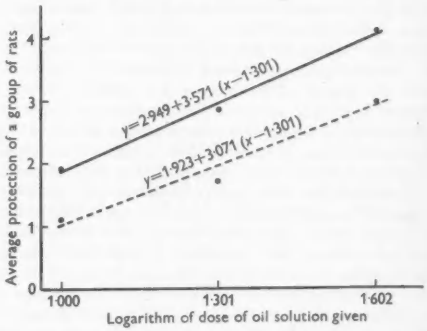


Fig. 1. Curve relating the degrees of protection of the central nervous system in male and female rats to the logarithm of the dose of vitamin A. ——, females; - - -, males.

Table 2. *Estimated degree of protection against myelin degeneration in female rats given by different doses of vitamin A*

(See sub-heading to Table 1.)

		Dose					
		0·2 i.u./day		0·4 i.u./day		0·8 i.u./day	
Exp. no.	Preparation	No. females	D.P.	No. females	D.P.	No. females	D.P.
1	Internat. Std. (*a*)	6	6·0	5	17·0	5	21·0
	Vaalhaai liver oil (*a*)	6	12·0	5	10·5	5	20·5
2	Internat. Std. (*b*)	5	4·0	4	8·5	4	15·5
	Vaalhaai liver oil (*b*)	5	5·5	4	4·5	4	8·5
3	Vit. A acetate (*a*)	8	21·0	6	21·5	6	24·5
	Internat. Std. (*a*)	8	25·5	6	20·5	6	23·5
	Vit. A distillate (*a*)	8	23·5	6	18·0	6	25·5
4	Vit. A acetate (*b*)	5	11·0	5	17·5	5	22·0
	Internat. Std. (*b*)	5	14·5	5	19·5	5	22·0
	Vit. A distillate (*b*)	5	16·5	5	18·0	5	26·0
5	Snoek liver oil (*a*)	6	11·0	6	15·5	5	12·0
	Vit. A acetate Std. (*a*)	6	8·0	5	12·5	5	21·0
	Hake liver oil (*a*)	6	10·5	6	19·5	5	20·5
6	Snoek liver oil (*b*)	4	3·5	4	9·5	5	20·0
	Vit. A acetate Std. (*b*)	4	6·0	5	5·5	5	22·0
	Hake liver oil (*b*)	4	9·0	4	11·0	5	23·5
7	Tuna liver oil (*a*)	5	12·0	5	13·5	5	22·5
	Internat. Std. (*a*)	5	14·0	5	17·5	5	21·5
	Dogfish liver oil (*a*)	5	8·5	5	15·5	4	17·5
8	Tuna liver oil (*b*)	5	7·5	5	13·5	5	22·0
	Internat. Std. (*b*)	5	7·5	5	15·5	5	18·5
	Dogfish liver oil (*b*)	5	8·0	5	11·5	5	18·5
9	Stonebass liver oil (*a*)	5	2·0	6	17·5	5	20·5
	Vit. A acetate Std.	6	12·0	5	13·5	6	25·5
	Stonebass liver oil (*b*)	6	8·5	5	10·0	6	25·5
	Vaalhaai liver oil (*c*)	6	9·0	6	13·0	4	13·0
	Totals	144	276·5	133	380·0	131	533·0
	Weighted means		1·920		2·857		4·069

lose vitamin A faster than those of females. There is, however, no sex difference with respect to method of utilization, as judged from the histologic picture.' These observations are in accord with the results obtained with male and female rats as demonstrated in Tables 1 and 2.

The dosing solutions were made up so as to contain approximately 0·6 i.u. of vitamin A per 10 mg. When dosing every 3 days, the 0·2 i.u. per day group of rats received 10 mg. of this oily solution, while the 0·4 and 0·8 i.u. per day groups were given 20 and 40 mg., respectively. The degrees of protection of the individual rats were added together and appear in columns 4, 6 and 8.

By plotting the average results obtained in Tables 1 and 2 against the logarithms (to the base 10) of the doses the curves of response proved to be straight lines (see Fig. 1).

The average protection of females against degeneration was, however, 2·949/1·923 = 1·533 times that of the males and it was, therefore, justifiable to assume that the minimum vitamin A requirement of male rats (about 9 weeks of age) was about 1·5 times that of females of the same age. Several workers have shown that the vitamin A requirement is a function of the body weight (Guilbert & Hart, 1935; Hart, 1940; Callison & Knowles, 1945; Paul & Paul, 1946). However, the latter alone cannot account for the sex difference in the daily requirement of this vitamin, or vice versa for the sex difference of protection against degeneration, since it is evident from the figures in Table 3 that the ratio of the body weights of male and female rats bears no relation to the protection ratio.

Curve of response. The sex difference in the response of rats to the same dose of vitamin A created a difficulty. It was impossible to use one sex only owing to the limitations in the breeding facilities. Two alternatives to solve the problem remained, namely, to keep the females longer on experiment until they reached a degeneration stage equal to that of the males, or to use equal numbers of males and females in all the groups. The former possibility was considered impracticable owing to the fact that it took more than 3 weeks to determine the stage of degeneration. With limited numbers of rats the latter alternative could not be followed.

To overcome this trouble of sex difference and to obtain results which might lend themselves readily to statistical analysis, Prof. S. J. Pretorius of Stellenbosch University suggested personally that an average factor should be calculated for each experi-

Table 3. *Average weights of rats at the end of the test period*

	Dose					
	0·2 i.u./day		0·4 i.u./day		0·8 i.u./day	
Preparation	Males (g.)	Females (g.)	Males (g.)	Females (g.)	Males (g.)	Females (g.)
Internat. Std. (*a*)	99	85	112	89	108	90
Vaalhaai liver oil (*a*)	101	84	111	87	102	93
Internat. Std. (*b*)	86	83	92	87	109	89
Vaalhaai liver oil (*b*)	84	85	97	84	113	87
Vit. A acetate (*a*)	135	105	140	99	151	116
Internat. Std. (*a*)	135	108	145	104	143	110
Vit. A distillate (*a*)	115	107	142	108	155	109
Vit. A acetate (*b*)	74	74	79	73	91	74
Internat. Std. (*b*)	70	73	80	70	94	82
Vit. A distillate (*b*)	72	76	78	77	98	74
Snoek liver oil (*a*)	81	86	100	83	113	85
Vit. A acetate Std. (*a*)	75	85	102	79	91	103
Hake liver oil (*a*)	85	82	95	86	103	88
Snoek liver oil (*b*)	88	69	94	83	109	90
Vit. A acetate Std. (*b*)	91	81	100	90	112	94
Hake liver oil (*b*)	93	79	91	84	105	87
Tuna liver oil (*a*)	100	80	102	85	111	88
Internat. Std. (*a*)	96	84	100	92	112	85
Dogfish liver oil (*a*)	99	81	109	89	112	88
Tuna liver oil (*b*)	91	81	99	84	106	94
Internat. Std. (*b*)	100	86	96	95	107	95
Dogfish liver oil (*b*)	93	81	105	89	111	99
Stonebass liver oil (*a*)	75	84	91	89	106	90
Vit. A acetate Std.	90	81	95	85	113	96
Stonebass liver oil (*b*)	88	82	84	89	114	81
Vaalhaai liver oil (*c*)	85	80	86	91	121	93
Totals	2441	2179	2626	2271	2910	2380
Averages	**94**	**84**	**101**	**87**	**112**	**91**
Ratio males/females	1·12		1·16		1·22	

ment by which to multiply the response of every individual female in that assay, so as to obtain a response corresponding to that of a male or vice versa. Such a factor represents the ratio of average response of males to average response of females in the present experiments. This suggestion was tested out and gave satisfactory results. Such transformed results were used in calculating the vitamin A potencies of the different oils.

Table 4. *Estimated protection against myelin degeneration given by different doses of International Standard β-carotene*

(Protection is expressed in terms of the results on single rats, transformed as indicated in text to eliminate sex differences.)

	Daily doses of International Standard		
	0·2 i.u. D.P.	0·4 i.u. D.P.	0·8 i.u. D.P.
	1·0	1·5	3·5
	1·0	3·0	3·0
	0·5	1·5	3·5
	2·0	3·0	4·5
	1·5	2·5	4·5
	2·71	3·05	2·71
	0·68	3·05	2·37
	1·70	1·70	3·39
	3·50	1·70	3·05
	1·36	2·37	3·05
	1·0	0·0	3·0
	2·0	2·0	3·5
	0·5	2·5	3·5
	1·5	2·0	2·5
	2·0	2·0	3·0
	1·26	1·90	2·84
	0·32	1·90	1·26
	1·26	0·95	2·52
	0·95	2·84	2·84
	0·95	2·21	2·21
Totals	27·24	41·67	60·74
Weighted means	**1·362**	**2·0835**	**3·037**

The log dose-response curve with this method was a straight line both for the β-carotene standard (Table 4) and vitamin A-containing oils (Table 5). The data of these two tables were used to plot the graphs in Fig. 2.

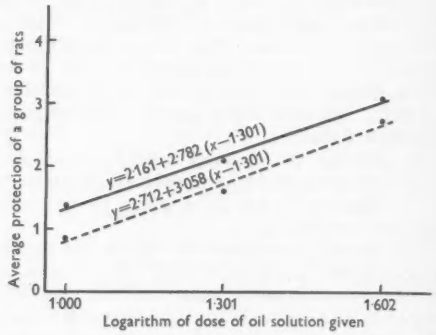


Fig. 2. Curve relating the degrees of protection of the central nervous system to the logarithm of the doses of β-carotene standard and fish-liver oils. ——, β-carotene standard; - - -, fish-liver oils.

Owing to the fact that the response of rats to a given dose of vitamin A is subject to fluctuations, 'it is absolutely essential that each test should be a comparison between the response given to a dose of the test substance and the response given to a dose of the standard *at the same time*' (Coward, 1947, p. 2). Experimental rats must be distributed in equal numbers between the biological standard and the substances to be tested and the slope of the response curve must be obtained from every individual test

Table 5. *Estimated degree of protection against myelin degeneration given by different doses of vitamin A*

	Dose					
	0·2 i.u.		0·4 i.u.		0·8 i.u.	
Oil sample	No. of rats	Value for group	No. of rats	Value for group	No. of rats	Value for group
Snoek (*a*)	10	9·76	10	16·34	10	24·96
Vit. A acetate Std. (*a*)	10	6·05	10	16·61	9	21·95
Hake (*a*)	9	7·47	10	18·63	10	28·18
Snoek (*b*)	10	6·17	10	15·89	10	22·93
Vit. A acetate Std. (*b*)	10	9·22	10	15·62	10	32·17
Hake (*b*)	10	12·58	10	16·82	10	29·62
Tuna (*a*)	10	14·15	9	21·15	10	35·25
Dogfish (*a*)	9	10·27	10	19·01	9	30·86
Tuna (*b*)	10	7·23	10	19·03	10	28·37
Dogfish (*b*)	10	9·05	9	11·75	10	22·67
Stonebass (*a*)	10	2·58	10	11·39	10	24·02
Vit. A acetate Std.	12	10·96	11	11·39	10	24·02
Stonebass (*b*)	10	7·09	10	13·89	10	21·71
Vaalhaai (*c*)	10	6·86	10	12·00	9	19·49
Totals	140	119·44	139	220·88	139	374·39
Weighted means		**0·8531**		**1·5891**		**2·6934**

itself. The slope from a previously constructed dosage-response curve cannot be used, because 'large changes of slope may occur with time, and... these changes do not occur in a random fashion but exhibit a secular or quasiperiodic tendency. The use of a fixed slope therefore introduces an uncontrolled error for which it is impossible to allow. Even if regular records of slopes are kept, and their standard deviation over a considerable period of time calculated, this does not get over the difficulty. For the variations are not random, and so this standard deviation gives no clue to the error of the slope at any particular time' (Irwin, 1944). This author demonstrated further that it is faulty to take the position and slope of the response curve given by the doses of vitamin A standard as correct and to use that for calculating the potency of the test substance. The correct procedure is to find the slope of the best fitting straight line through all the observed points; 'that is to say, it assumes that apart from sampling error the slopes provided by the standard and the test substance are the same. The horizontal distance between the parallel straight lines then provides an estimate of the potency ratio.'

A similar method of calculation was used in this work and Table 6 illustrates the fluctuations which were found in the slopes of the response curves.

The slopes found for every section show very good agreement, but significant differences exist between slopes of different assays, e.g. section no. 3 and section no. 6. These data therefore confirm the statement of Coward quoted above.

As the log dose-response curve is a straight line, the statistical method described in the British Standards Institution Specification no. 911 (1940) was used to analyse the experimental data and the results are summarized in Table 7. A comparison of the limits of error in this table and those given by Gridgeman (1944) and Irwin (1937) for the 3 weeks' curative 'growth' method indicates that this histological method for the bio-assay of vitamin A is as accurate as and possibly more accurate than the 3 weeks' curative 'growth' test.

Table 6. *Fluctuations in the slopes of the log dose-response curves*

Section no.	Test oil	– Standard	Test period	Slope *
1	Vaalhaai (*a*)	– Internat. Std.	2. iii. 47–18. iv. 47	0·786
2	Vaalhaai (*b*)	– Internat. Std.	24. iii. 47–6. v. 47	0·475
3	Vit. A acetate (*a*)	– Internat. Std.	27. iv. 47–29. vi. 47	0·563
	Vit. A distillate (*a*)	– Internat. Std.	27. iv. 47–29. vi. 47	0·547
4	Vit. A acetate (*b*)	– Internat. Std.	1. vi. 47–14. vii. 47	0·635
	Vit. A distillate (*b*)	– Internat. Std.	1. vi. 47–14. vii. 47	0·521
5	Snoek (*a*)	– Vit. A acetate Std.	21. vi. 47–11. viii. 47	0·838
	Hake (*a*)	– Vit. A acetate Std.	21. vi. 47–11. viii. 47	0·956
6	Snoek (*b*)	– Vit. A acetate Std.	21. vii. 47–7. ix. 47	0·993
	Hake (*b*)	– Vit. A acetate Std.	21. vii. 47–7. ix. 47	1·000
7	Tuna (*a*)	– Internat. Std.	12. viii. 47–25. ix. 47	0·954
	Dogfish (*a*)	– Internat. Std	12. viii. 47–25. ix. 47	1·017
8	Tuna (*b*)	– Internat. Std.	14. ix. 47–23. x. 47	0·914
	Dogfish (*b*)	– Internat. Std.	14. ix. 47–23. x. 47	0·726
9	Stonebass (*a*)	– Vit. A acetate Std.	31. x. 47–12. xi. 47	0·970
	Stonebass (*b*)	– Vit. A acetate Std.	31. x. 47–12. xi. 47	0·815
	Vaalhaai (*c*)	– Vit. A acetate Std.	31. x. 47–12. xi. 47	0·819

* The values of these slopes are not of the same magnitude as those previously used.

Table 7. *Limits of error and conversion factors estimated for different vitamin A preparations*

(The average number of rats used in an assay of one oil was 30.)

Oil sample	Conversion factor	Average conversion factor	Limits of error (%)
Vaalhaai (*a*)	1805	—	84·2–118·9
(*b*)	1659	—	51·1–185·3
(*c*)	1391	1618	74·3–130·9
Vit. A acetate (*a*)	1818	—	52·6–151·2
(*b*)	1774	1796	54·6–179·5
Vit. A distillate (*a*)	1455	—	63·6–156·2
(*b*)	2459	1957	67·9–292·2
Tuna (*a*)	1776	—	75·6–132·1
(*b*)	1684	1730	78·4–126·8
Dogfish (*a*)	1557	—	75·1–130·4
(*b*)	1265	1411	69·4–135·8
Snoek (*a*)	1788	—	75·9–134·0
(*b*)	1234	1511	71·8–134·0
Hake (*a*)	1951	—	73·5–141·6
(*b*)	1683	1817	75·7–133·0
Stonebass (*a*)	1408	—	78·9–124·5
(*b*)	1538	1473	77·0–128·0

The given doses of both the vitamin A standard and the test oil were always in geometrical progression. This did not apply to the total vitamin A intake, owing to the fact that the rats received small but equal amounts of carotene derived from the

potato starch. Such an extra allowance, although small, would obviously tend to interfere with the log dose-response relationship. Therefore, a more satisfactory vitamin A-free diet would probably increase the accuracy of this method.

Correction for irrelevant absorption. Until recently it was believed that the full intensity of absorption at 328 mμ. was due to the presence of vitamin A. This assumption was proved to be unjustified, owing to the presence of irrelevant absorbing substances, even in high-potency oils and their unsaponifiable fractions.

The influence of the correction for irrelevant absorption, as suggested by Morton & Stubbs (1946, 1948) on the conversion factors obtained in this work, is illustrated in Table 8.

Table 8. *Influence of correction for irrelevant absorption on the conversion factor*

Preparation	Average* potency (i.u./g.)	*E*		Conversion factor	
		Observed	Corrected	Observed	Corrected
Vaalhaai liver oil	15,000	9·26	8·68	1618	1727
Vit. A acetate	12,300	6·85	6·80	1796	1810
Vit. A distillate	16,500	8·44	8·15	1957	2028
Tuna liver oil	129,900	75·10	67·50	1730	1925
Dogfish liver oil	5,710	4·05	3·60	1411	1586
Snoek liver oil	26,700	17·64	16·56	1511	1610
Hake liver oil	13,900	7·68	7·25	1817	1924
Stonebass liver oil	78,600	53·37	46·75	1473	1682
			Averages	1665†	1786

* International β-carotene preparation accepted as primary standard throughout.
† The average value for the liver oils only was 1593.

It is evident from the data in Table 8 that a conversion factor of 1600 is preferable for uncorrected *E* values and that the conversion factor of 2000 which is generally used in America, is definitely too high. A higher conversion factor should be used for corrected *E* values and the value of 1800 suggested by Morton & Stubbs (1948) is supported by these data.

DISCUSSION

The main objection to the use of a prophylactic method is that it takes longer to perform than the curative test. Time, labour and money, however, are saved at the feeding stage of this assay, seeing that the test period is not longer than 6 weeks, whereas the depletion period of the curative method takes about 5 weeks and the test period is at least 3 weeks in addition. Thus, the experimental periods for this prophylactic method and the curative test are 6, and at least 8 weeks, respectively.

The curative method has the further disadvantage that frequently rats have to be rejected at the end of the preliminary period, because their weight curves render them 'unsuitable' for the test. A further reduction in the number of available animals may result owing to losses by death during the depletion period. The curative method is also carried out on animals which may differ widely in pathological condition, and this main source of error is avoided by using the prophylactic method. In this bio-assay no animals need to be discarded.

SUMMARY

1. Degeneration in the central nervous system is a characteristic symptom of vitamin A deficiency in rats. The Marchi technique is sufficiently accurate for following such degenerative changes in the myelin substance of the central nervous system, provided that it is properly used. With slight modifications in histological procedure it can be used for quantitative routine work.

2. A histological method for the biological estimation of vitamin A is described. If groups of young rats are kept under the same experimental conditions and receive graded doses of vitamin A over a suitable range, equal proportional increases in doses produce approximately equal average increases in protection against degeneration in the central nervous system.

3. There exists a significant difference between the response (protection against degeneration) of male and female rats to the same dose of vitamin A. This difference indicates that the minimum vitamin A requirement of male rats is about 1·5 times that of females of the same age. Although it is generally assumed that vitamin A requirement is a function of body weight, the latter alone cannot account for the sex difference.

4. The vitamin A potencies of vitamin A acetate, vitamin A distillate and six natural fish-liver oils were estimated by means of this histological method and corresponding conversion factors were calculated to test out the reliability of the present conversion factors.

5. The experimental data were analysed statistically with the result that the histological method seemed to be as accurate as, and possibly more accurate than, the 3 weeks' curative 'growth' test.

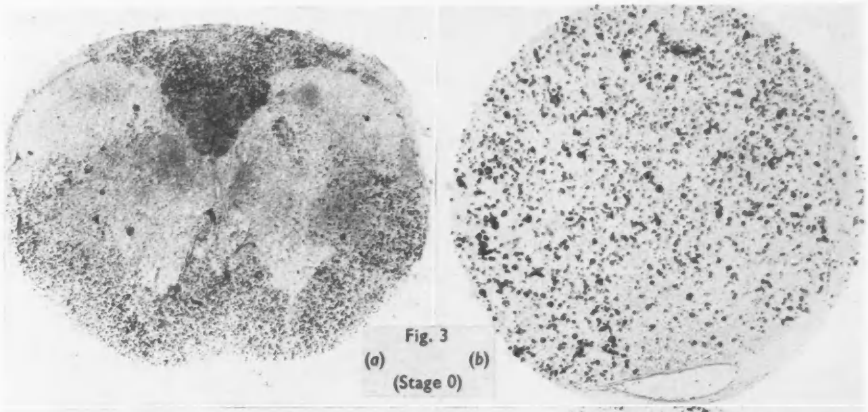


Fig. 3
(*a*) (*b*)
(Stage 0)

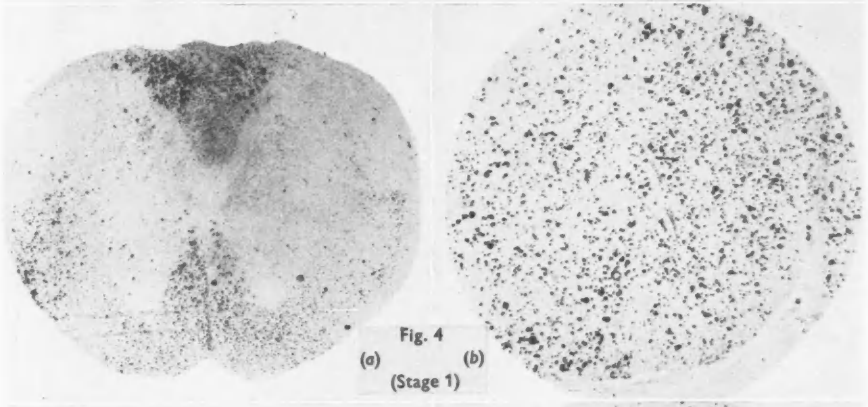


Fig. 4
(*a*) (*b*)
(Stage 1)

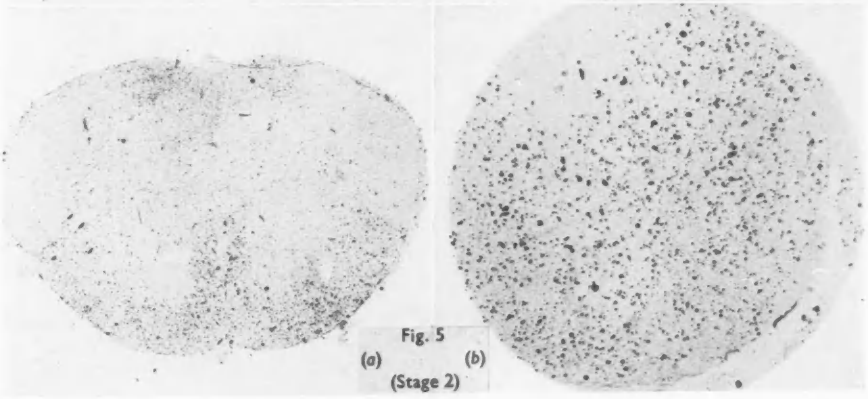


Fig. 5
(*a*) (*b*)
(Stage 2)

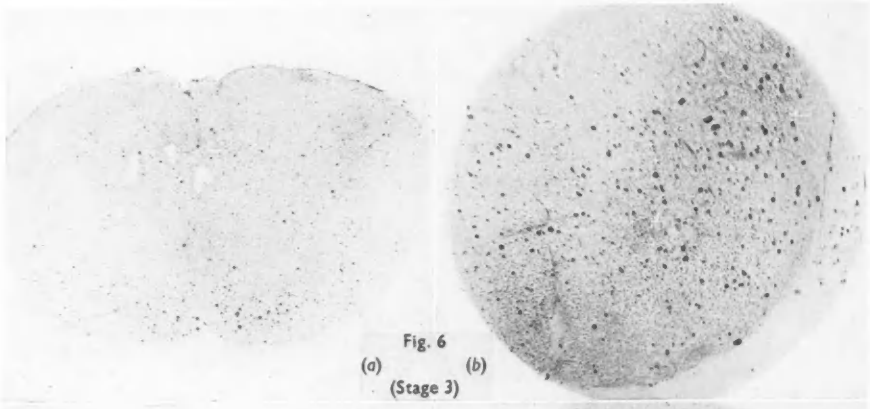


Fig. 6
(*a*) (*b*)
(Stage 3)

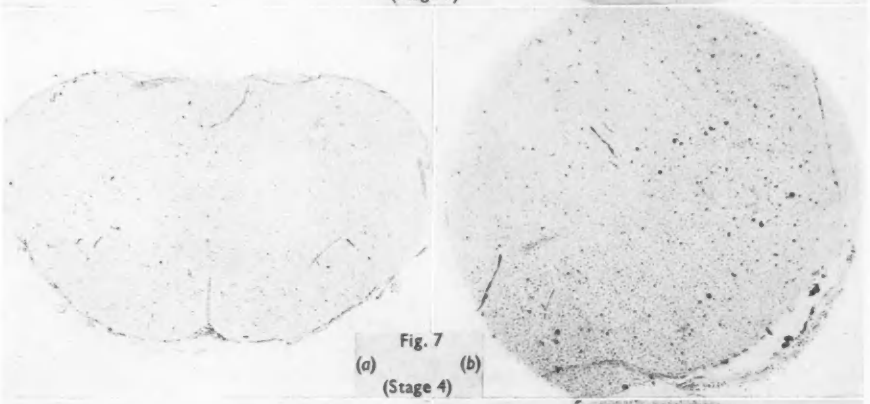


Fig. 7
(*a*) (*b*)
(Stage 4)

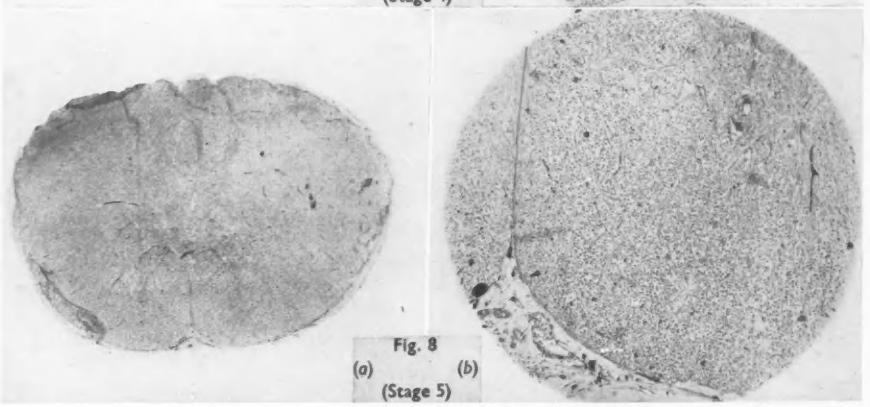


Fig. 8
(*a*) (*b*)
(Stage 5)

6. The calculated conversion factors indicate that 1600 is preferable to 2000 for uncorrected *E* values. A higher factor should be used for corrected *E* values.

The author wishes to express his gratitude to Prof. J. T. Irving for his encouragement, guidance and continued interest in this work; to Dr H. M. Schwartz for her highly esteemed advice and assistance; to Prof. S. J. Pretorius for his advice in the carrying out of the statistical analysis; to Mr H. Hall for taking the photomicrographs.

The author was awarded a research assistantship by the Council of Scientific and Industrial Research of South Africa to enable him to carry out this work; for this, grateful acknowledgement is made. The expenses of the research were also borne by the same body.

REFERENCES

Aberle, S. B. D. (1934). *J. Nutrit.* **7**, 445.
Brenner, S., Brookes, M. C. H. & Roberts, L. J. (1942). *J. Nutrit.* **23**, 459.
British Standards Institution Specification no. 119 (1940). London: British Standards Institution.
Callison, E. C. & Knowles, V. H. (1945). *Amer. J. Physiol.* **143**, 444.
Copp, D. H. & Greenberg, D. M. (1945). *J. Nutrit.* **29**, 261.
Coward, K. H. (1932). *Biochem. J.* **26**, 691.
Coward, K. H. (1947). *The Biological Standardisation of the Vitamins*, 2nd ed. London: Baillière, Tindall and Cox.
Eveleth, D. F. & Biester, H. E. (1937). *Amer. J. Path.* **13**, 257.
Gridgeman, N. T. (1944). *The Estimation of Vitamin A.* London: Lever Brothers and Unilever, Ltd.
Guilbert, H. R. & Hart, G. H. (1935). *J. Nutrit.* **10**, 409.
Hart, E. B. & McCollum, E. V. (1914). *J. biol. Chem.* **19**, 373.
Hart, E. B., Miller, W. S. & McCollum, E. V. (1916). *J. biol. Chem.* **25**, 239.
Hart, G. H. (1940). *Nutrit. Abstr. Rev.* **10**, 261.
Hughes, J. S., Lienhardt, H. F. & Aubel, C. E. (1929). *J. Nutrit.* **2**, 183.
Irving, J. T. (1948). *Nature, Lond.*, **162**, 377.
Irving, J. T. & Richards, M. B. (1937). *J. Physiol.* **89**, 2*P*.
Irving, J. T. & Richards, M. B. (1938). *J. Physiol.* **94**, 307.
Irving, J. T. & Richards, M. B. (1940). *Biochem. J.* **34**, 198.
Irwin, J. O. (1937). *J. R. statist. Soc.* Suppl. **4**, 1.
Irwin, J. O. (1944). *J. Hyg., Camb.*, **43**, 291.
Lee, J. & Sure, B. (1937). *Arch. Path.* **24**, 430.
Mellanby, E. (1926). *J. Physiol.* **61**, xxiv.
Mellanby, E. (1930). *Brit. med. J.* i, 677.
Mellanby, E. (1931). *J. Amer. med. Ass.* **96**, 325.
Mellanby, E. (1934). *J. Path. Bact.* **38**, 391.
Mellanby, E. (1938). *J. Physiol.* **93**, 42*P*.
Mellanby, E. (1941). *J. Physiol.* **99**, 467.
Mellanby, E. (1947). *J. Physiol.* **105**, 382.
Moore, L. A. & Sykes, J. F. (1940). *Amer. J. Physiol.* **130**, 684.
Moore, L. A. & Sykes, J. F. (1941). *Amer. J. Physiol.* **134**, 436.
Morton, R. A. (1942). *Ann. Rev. Biochem.* **11**, 365.
Morton, R. A. & Stubbs, A. L. (1946). *Analyst*, **71**, 348.
Morton, R. A. & Stubbs, A. L. (1948). *Biochem. J.* **42**, 195.
Orr, J. B. & Richards, M. B. (1934). *Biochem. J.* **28**, 1259.
Paul, H. E. & Paul, M. F. (1946). *J. Nutrit.* **31**, 67.
Popper, H. & Brenner, S. (1942). *J. Nutrit.* **23**, 431.
Setterfield, H. E. & Sutton, T. S. (1935). *J. Nutrit.* **9**, 645.
Suzman, M. M., Muller, G. L. & Ungley, C. C. (1932). *Amer. J. Physiol.* **101**, 529.
Wolbach, S. B. & Bessey, O. A. (1940). *Science*, **91**, 599.
Wolbach, S. B. & Bessey, O. A. (1941). *Arch. Path.* **32**, 689.
Wolbach, S. B. & Bessey, O. A. (1942). *Physiol. Abstr.* 212.
Wolbach, S. B. & Howe, P. R. (1925). *J. exp. Med.* **42**, 753.
Zimmerman, H. M. (1933). *J. exp. Med.* **57**, 215.
Zimmerman, H. M. & Cowgill, G. R. (1936). *J. Nutrit.* **11**, 411.

EXPLANATION OF PLATES 6 AND 7

Cross-sections of the medulla showing different stages of protection against degeneration.

Marchi-stained sections of the cord do not lend themselves readily to photography, especially at lower magnifications. The contrast of the normal photomicrograph is insufficient, especially for comparisons of the lower stages of degeneration. To overcome this trouble and to produce a greater degree of contrast, it was found necessary to transpose the negatives, which decreased the detail of the undegenerated structure and increased that of the degenerated tracts.

Figs. 3–8 show the six stages of protection against degeneration; (*a*) in each case, shows the entire spinal cord (×14) and (*b*) the corresponding anterolateral column (×45), where the degeneration on which the classification was based chiefly occurred.

It is evident from these photomicrographs that the first degeneration in vitamin A deficiency occurs in the funiculus praedorsalis and develops gradually in the columns of Goll and Burdach. Even in the second stage of protection the posterior columns are affected to only a very slight degree in comparison with the degeneration in the anterolateral columns. However, the severity of degeneration in the posterior columns increases rapidly in the later stages and reaches the same degree as in the anterior columns when there is no protection.

Studies in Detoxication

28. THE BIOSYNTHESIS OF *o*-, *m*- AND *p*-CYANOPHENYLGLUCURONIDES

By J. N. SMITH
Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2

(*Received* 3 *August* 1949)

Studies in progress in this laboratory on the fate of cyanobenzene in the rabbit (Smith & Williams, 1949) required as reference compounds the glucuronides of the *o*-, *m*- and *p*-cyanophenols. These compounds were made biosynthetically by feeding the necessary phenols to rabbits and isolating the glucuronides from the urine. Only *p*-cyanophenylglucuronide was isolated as such, the *o*- and *m*-glucuronides being obtained as crystalline amides and triacetyl methyl esters.

EXPERIMENTAL

o-*Cyanophenylglucuronide*

o-Cyanophenol (1·5 g.; m.p. 90°)* was fed in aqueous suspension to a 3·5 kg. rabbit. No ill effects were noted, and after 4 hr. the urine (70 ml.) was collected. The urine gave negative tests with Benedict reagent, $FeCl_3$ and dichloroquinonechloroimide, but an intense test with naphthoresorcinol. The basic Pb acetate precipitate of the urine was prepared as described in earlier papers (e.g. Smith & Williams, 1948). The Pb was removed with H_2S and the aqueous residue taken to a gum *in vacuo*. This gum (*o-cyanophenylglucuronide*) was soluble in 95% ethanol and water, but attempts to crystallize it or to form crystalline salts with benzylamine or triethanolamine were not successful. Urine collected after 4 hr. contained only traces of the glucuronide. The gum was dissolved in 5 ml. 95% ethanol and methylated by shaking for an hour with an excess of diazomethane in 150 ml. diethyl ether. The mixture was filtered and the ether removed *in vacuo* leaving a neutral gum which was presumably the *methyl ester* of *o*-cyanophenylglucuronide. This ester was now dissolved in a mixture of 10 ml. acetic anhydride and 10 ml. pyridine. After 20 hr. at room temperature, the mixture was diluted to 100 ml. with water and cooled to 0°. Crystals (1·35 g. or 25% of the dose) of the *methyl ester of triacetyl β-o-cyanophenyl-D-glucuronide* were thrown out. These were collected and recrystallized from absolute ethanol-ligroin as long, colourless needles, m.p. 151–152° and $[\alpha]_D^{20°}$ – 71° (*c*, 4·9 in $CHCl_3$). (Found: C, 55·45; H, 4·8; N, 3·4; OMe, 7·2. $C_{20}H_{21}O_{10}N$ requires C, 55·2; H, 4·9; N, 3·2; OMe, 7·1%.) The compound was sparingly soluble in hot water, hot ligroin and CCl_4, easily soluble in $CHCl_3$, acetone, ethanol and ethyl acetate and insoluble in light petroleum.

β-o-Cyanophenyl-D-glucuronidamide. The above acetylated ester (0·1 g.) was dissolved in 5 ml. absolute ethanol, and the solution saturated with NH_3 at 0°. On standing overnight *β-o-cyanophenyl-D-glucuronidamide hemihydrate* was deposited as fine colourless needles. It was recrystallized from ethanol/light petroleum and had m.p. 195° and $[\alpha]_D^{20°}$ – 78° (*c*, 1·3 in 50% aqueous ethanol). (Found: C, 51·3; H, 5·0; N, 9·7; $C_{13}H_{14}O_6N_2.0{\cdot}5\ H_2O$ requires C, 51·5; H, 5·0; N, 9·5%.) The compound was soluble in water, sparingly soluble in ethanol and insoluble in ether and light petroleum.

* All melting points are uncorrected.

m-*Cyanophenylglucuronide*

m-Cyanophenol (2 g.; m.p. 83°) was fed, as a suspension in water, to a 3 kg. rabbit and the clear yellow 4 hr. urine (100 ml.), which gave only the naphthoresorcinol test, was worked up as for the *o*-compound. *m-Cyanophenylglucuronide* was obtained as a gelatinous solid which dried to a non-crystalline powder soluble in 95% ethanol and water. The powder was methylated in ethanol for 3 hr. with excess ethereal diazomethane to give a gummy *methyl ester*. This ester was acetylated as above and yielded 2·9 g. (40% of dose) of the crystalline *methyl ester of triacetyl-β-m-cyanophenyl-D-glucuronide*. Its solubility was similar to that of the *o*-compound. On recrystallization from absolute ethanol the compound formed colourless needles, m.p. 156–157° and $[\alpha]_D^{20°}$ – 37° (*c*, 3·2 in $CHCl_3$). (Found: C, 55·3; H, 5·2; N, 3·7; OCH_3, 7·2. $C_{20}H_{21}O_{10}N$ requires C, 55·2; H, 4·9; N, 3·2; OCH_3, 7·1%.)

β-m-Cyanophenyl-D-glucuronidamide was obtained by treating the above ester (0·25 g.) in ethanol (4 ml.) with NH_3, and after keeping the solution overnight the amide (0·1 g.) was precipitated from the solution by ether. On recrystallization from hot ethanol, the *amide* formed colourless needles of the monohydrate, m.p. 207° (decomp.) and $[\alpha]_D^{23°}$ – 63° (*c*, 0·4 in water). (Found: C, 50·4; H, 5·3; N, 9·5. $C_{13}H_{14}O_6N_2.H_2O$ requires C, 50·0; H, 5·2; N, 9·0%.) It was soluble in water and ethanol but not in benzene or ether.

p-*Cyanophenylglucuronide*

p-Cyanophenol (1·5 g., m.p. 110°) was fed to a 3 kg. rabbit as before. A 4 hr. urine (100 ml.) was collected which gave no colour with $FeCl_3$, no reduction with Benedict reagent but an intense naphthoresorcinol test. On extracting the untreated urine with ether, an extract was obtained which gave feebly, with $FeCl_3$, the colours characteristic of a catechol derivative. The glucuronide fraction of the urine was separated by the Pb acetate procedure and the Pb-free filtrate was taken down *in vacuo* to a thin syrup, which solidified on standing. The solid was treated with a little water and the crystals (230 mg. or 6% of the dose) of *p-cyanophenylglucuronide monohydrate* filtered off. The compound on recrystallization from water formed colourless needles, m.p. 140° (decomp.) and $[\alpha]_D^{20°}$ – 92° (*c*, 3·5 in

Table 1. *Melting points and optical rotations of biosynthetic cyanophenylglucuronides and derivatives*

	Glucuronide		Triacetyl methyl ester of glucuronide		Glucuronidamide	
Cyanophenol	M.p.	$[\alpha]_D^{20°}$ in water	M.p.	$[\alpha]_D^{20°}$ in $CHCl_3$	M.p.	$[\alpha]_D^{20°}$ in water
ortho-	Not crystallized		151°	−71°	195°*	−78°†
meta-	Not crystallized		156–157°	−37°	207°‡	−63°
para-	140°‡	−92°	130–135°	−42°	210–213°	−72°

* Hemihydrate. † In 50% aqueous ethanol. ‡ Monohydrates.

water). (Found: C, 50·15; H, 4·9; N, 4·45. $C_{13}H_{13}O_7N, H_2O$ requires C, 49·85; H, 4·8; N, 4·5%.) The glucuronide was soluble to the extent of 5% in water; it was more soluble in ethanol but insoluble in ether.

The mother liquors after the separation of the crystalline glucuronide were evaporated to dryness, and the residue dissolved in a little 95% ethanol. The solution, on treatment with ethereal diazomethane as before, gave a gummy *methyl ester*. The latter, on acetylation with pyridine and acetic anhydride for 48 hr. at room temperature and then pouring into water, yielded the crystalline *methyl ester* of *triacetyl β-p-cyanophenyl-D-glucuronide* (0·51 g. or 9% of the dose). On recrystallization from ethanol-light petroleum the compound formed colourless needles, m.p. 130–135° and $[\alpha]_D^{20°}$ −42° (*c*, 1·4 in $CHCl_3$). (Found: C, 54·8; H, 4·75; N, 3·65. $C_{20}H_{21}O_{10}N$ requires C, 55·2; H, 4·9; N, 3·2%.)

β-p-Cyanophenyl-D-glucuronidamide. Treatment of the above acetylated ester (0·3 g.) with ethanol saturated with NH_3 at 0°, yielded after keeping overnight no crystalline material. However, on diluting the solution with light petroleum, the *amide* was thrown out as an oil which gradually solidified (yield 0·15 g.). It was recrystallized from ethanol-benzene and formed fine colourless needles, m.p. 210–213° (decomp.) and $[\alpha]_D^{20°}$ −72° (*c*, 2·6 in water). (Found: C, 53·3; H, 5·1; N, 9·5. $C_{13}H_{14}O_6N_2$ requires C, 53·05; H, 4·8; N, 9·5%.)

DISCUSSION

The properties of the *o-*, *m-* and *p*-cyanophenylglucuronides and their derivatives are summarized in Table 1.

The only figure calling for comment is the specific optical rotation of the methyl ester of triacetyl *o*-cyanophenylglucuronide which is nearly twice that of its isomers. The rotations of the three amides are, however, of the same order. It is known that the tetra-acetates of some *ortho*-substituted phenyl-*β*-glucosides have anomalous rotations (see Pigman & Goepp, 1948) when compared with the *m-* and *p*-isomers. In view of this, the high negative rotation of triacetyl *o*-cyanophenyl-*β*-glucuronide methyl ester compared with the *m-* and *p*-isomers is not unexpected. It may be noted also that the methyl ester of triacetyl *β-o*-methoxyphenylglucuronide (Garton & Williams, 1948, 1949) has $[M]_D$ − 21,560 in ethanol, while the corresponding derivatives of *β-m*-acetoxyphenyl- and *β-p*-acetoxyphenyl-glucuronides have $[M]_D$ values about half of this (− 11,466 and − 10,296, respectively) in acetone.

One further point in this work is worthy of comment and that is that the main bulk of the cyanophenylglucuronides was excreted within 4 hr. of administering the cyanophenols, none of which was toxic at 0·5 g./kg.

SUMMARY

1. The preparation of *p*-cyanophenylglucuronide, by feeding *p*-cyanophenol to rabbits, is described.

2. The characterization of the biosynthetic *o-*, *m-* and *p*-cyanophenylglucuronides as their amides and triacetyl methyl esters, is described.

The author wishes to thank Prof. R. T. Williams for his stimulating interest and encouragement in this work.

REFERENCES

Garton, G. A. & Williams, R. T. (1948). *Biochem. J.* **43**, 206.
Garton, G. A. & Williams, R. T. (1949). *Biochem. J.* **44**, 234.
Pigman, W. W. & Goepp, R. M. (1948). *Chemistry of the Carbohydrates*, p. 88. New York: Academic Press.
Smith, J. N. & Williams, R. T. (1948). *Biochem. J.* **42**, 538.
Smith, J. N. & Williams, R. T. (1949). *Biochem. J.* (in the Press).

Index of Authors

Note. Entries marked with an asterisk (*) refer to communications made in title only.
Entries marked with a section mark (§) refer to contributions to symposia.
Small roman numerals refer to pages in *Proceedings of the Biochemical Society.*

Abdel Kader, M. M. *see* Ellinger, P. 276
Abul-Fadl, M. A. M. & King, E. J. Properties of the acid phosphatases of erythrocytes and of the human prostate gland 51
Ada, G. L. Phospholipin metabolism in rabbit-liver cytoplasm 422
Adair, G. S., Bailey, K. & Tsao, T. C. Effects of electrolytes on the molecular weight of tropomyosin v
Anderson, A. B. Inactivation of serum alkaline phosphatase by adrenaline and related substances xxxvi
Anderson, D. J. Nitrogen in human dental enamel 31
Arnstein, H. R. V. & Neuberger, A. The conversion of acetate to glycine in the rat iii
Aubert, X. *see* Nys, A. 245

Bacon, J. S. D. & Edelman, J. Carbohydrates present in the subterranean organs of some members of the Compositae xxviii
Bacon, J. S. D. *see also* Edelman, J. xxix
Bailey, K. The enzymic degradation of adenosinetriphosphate 479
Bailey, K. *see also* Adair, G. S. v
Ball, S., Collins, F. D., Dalvi, P. D. & Morton, R. A. Studies in vitamin A. 11. Reactions of retinene$_1$ with amino compounds 304
Ball, S. & Morton, R. A. Studies in vitamin A. 10. Vitamin A$_1$ and retinene$_1$ in relation to photopic vision 298
Bardawill, C. J. & Gornall, A. G. The influence of dietary protein levels on experimental liver injury in dogs iii
Barua, R. K. & Morton, R. A. Studies in vitamin A. 12. Whale-liver oil analysis: preparation of kitol esters 308
*Basil, B. Potency calculator for use with logarithmic dose response curves xvii
Beattie, J. & Corcoran, A. C. Observations on the renal clearance of some fructosans xv
Beaven, G. H. & Brown, F. The pectic enzymes of the mould *Byssochlamys fulva* 221
Beeckmans, M.-L. *see* Popják, G. x
Bell, D. J. & Palmer, A. Some observations on the structures of certain fructosans xiv
Bell, E. A., Cocker, W. & O'Meara, R. A. Q. The intracellular mode of action of the sulphonamide derivatives. Some condensation products of reductone 373
Bender, A. E., Krebs, H. A. & Horowitz, N. H. Amino-acid oxidase of *Neurospora crassa* xxi
Bentley, R. An apparatus for the micro-electrolysis of water 591
Bentley, R. & Neuberger, A. The mechanism of the action of notatin 584
Berridge, N. J. Preparation of the antibiotic nisin 486
Bhagvat, K. & Devi, P. Interrelationship of certain vitamins of the B group in aneurin, riboflavin and biotin deficiences 32
Blackburn, S. The use of buffered columns in the chromatographic separation of 2:4-dinitrophenyl amino-acids 579
Blewett, M. *see* Campbell, I. G. 105
Bone, A. D. *see* Weil-Malherbe, H. 377
Boughton, B. W. *see* Pollock, M. R. 417
Bowes, J. H. & Kenten, R. H. Some observations on the amino-acid distribution of collagen, elastin and reticular tissue from different sources 281
Brain, F. H. & Helliwell, P. J. The estimation of trichloroethylene in blood 75
Bray, H. G., Humphris, B. G. & Thorpe, W. V. Metabolism of derivatives of toluene. 3. *o*-, *m*- and *p*-Xylenes 241
Bray, H. G., James, S. P., Thorpe, W. V., Wasdell, M. R. & Wood, P. B. The fate of certain organic acids and amides in the rabbit. 9. Lower aliphatic amides 467
Bray, H. G., Thorpe, W. V. & Wood, P. B. The fate of certain organic acids and amides in the rabbit. 8. Toluic acids and amides 45
Brews, V. A. L. *see* Tárnoky, A. L. 508
Brown, F. *see* Beaven, G. H. 221
*Brown, M. B. *see* Cook, R. P. xvii
Brummel, E. *see* Early, D. F. 552
Butler, G. C. *see* Fleming, D. S. 546

Calam, C. T., Raistrick, H. & Todd, A. R. The potato eelworm hatching factor. 1. The preparation of concentrates of the hatching factor and a method of bioassay 513
Calam, C. T., Todd, A. R. & Waring, W. S. The potato eelworm hatching factor. 2. Purification of the factor by alkaloid salt fractionation. Anhydrotetronic acid as an artificial hatching agent 520
Cama, H. R. & Goodwin, T. W. Studies in vitamin A. 9. The role of the thyroid in carotene and vitamin A metabolism 236
Cama, H. R. & Goodwin, T. W. Studies in vitamin A. 13. The alleged formation of vitamin A from β-carotene treated with iodinated casein 317
Campbell, I. G., Olley, J. & Blewett, M. The effect of dietary oleic and palmitic acids on the composition and turnover rates of liver phospholipins 105
Chanda, R., McNaught, M. L. & Owen, E. C. The effect of thyroxine and thiouracil on some of the water-soluble vitamins in milk xix
Challenger, F. & Fothergill, P. Experiments on the mobility of the methyl group in dimethylacetothetin, $(CH_3)_2S^+.CH_2CO.O^-$ xxvii
Chattaway, F. W., Dolby, D. E., Hall, D. A. & Happold, F. C. Growth factors for *Corynebacterium diphtheriae*. 4. Preparation from yeast 592
Chen, Yu-Tuan *see* Mapson, L. W. 171
Chick, H. & Slack, E. B. Distribution and nutritive value of the nitrogenous substances in the potato 211
Clayton, J. C., Free, A. A., Page, J. E., Somers, G.F. & Woollett, E. A. Absorption and excretion of DL-thyroxine labelled with radioactive iodine xx
Cocker, W. *see* Bell, E. A. 373
Coetzee, W. H. K. A histological method for the biological estimation of vitamin A 628
Cohen, G. N. *see* Cohen-Bazire, G. 41
Cohen-Bazire, G. & Cohen, G. N. Direct transformation of fumarate to oxaloacetate, without intermediate formation of malate, by *Clostridium saccharobutyricum*, strain GR4 41
Collins, F. D. *see* Ball, S. 304
Comfort, A. Acid-soluble pigments of molluscan shells. 2. Pigments other than porphyrins 199
Comfort, A. Acid-soluble pigments of molluscan shells. 3. The indigoid character of the blue pigment of *Haliotis cracherodii* Leach. 204
Comfort, A. *see also* Nicholas, R. E. H. 208

COOK, E. R. *see* REISS, M. 574
*COOK, R. P. & BROWN, M. B. Chemical constituents of the pea (*Pisum sativum*) xvii
CORCORAN, A. C. *see* BEATTIE, J. xv
COXON, R. V. LIÉBECQ, C. & PETERS, R. A. The pyruvate-oxidase system in brain and the tricarboxylic acid cycle 320
CRANE, E. E. & DAVIES, R. E. Transport of radioactive Na^+ and K^+ through gastric mucosa xxiii
CROMWELL, B. T. The micro-estimation and origin of methylamine in *Mercurialis perennis* L. 84
CRUICKSHANK, E. M. *see* MAPSON, L. W. 171
*CUTHBERTSON, W. F. J. & LLOYD, J. T. Cup plate assay of vitamin B_{12} xvii
CUTHBERTSON, W. F. J. *see also* LESTER SMITH, E. xii

DAGLEY, S. & DAWES, E. A. Factors influencing the polysaccharide content of *Escherichia coli* 331
DAGLEY, S. & DAWES, E. A. Factors influencing the polysaccharide content of *Escherichia coli* xxvi
DAGLEY, S., DAWES, E. A. & MORRISON, G. A. The 'early-lag' phase of *Aerobacter aerogenes* xxv
DAGLEY, S., DAWES, E. A. & MORRISON, G. A. Influence of amino-acids, and compounds in the Krebs oxidation cycle, on 'early-lag' xxvi
DALE, W. M., DAVIES, J. V. & GILBERT, C. W. The kinetics and specificities of deamination of nitrogenous compounds by X-radiation 93
DALE, W. M., DAVIES, J. V. & GILBERT, C. W. The deamination of glycine by α-radiation from the disintegration of boron in a nuclear reactor 543
DALVI, P. D. *see* BALL, S. 304
DANIEL, R. J. The distribution of ascorbic acid in developing salmon (*Salmo salar* L.) 435
DAVIDSON, J. N. & LESLIE, I. The changing chemical composition of chick-heart explants grown *in vitro* xv
DAVIDSON, J. N. & MCINDOE, W. M. Phosphorus compounds in the cell nucleus xvi
DAVIES, A. W. *see* RODAHL, K. 408
DAVIES, C. W. The fractionation of weak electrolyte mixtures by ion-exchange resins 38
DAVIES, J. V. *see* DALE, W. M. 93, 543
DAVIES, R. E. *see* CRANE, E. E. xxiii
DAWES, E. A. *see* DAGLEY, S. 331, xxv, xxvi
DEAN, R. F. A. *see* MCCANCE, R. A. 493
DEVI, P. & HIGGINBOTTOM, C. Quantitative studies on mutants of *Bacterium aerogenes* requiring nicotinic acid and histidine 391
DEVI, P. *see also* BHAGVAT, K. 32
DOBSON, F. & STROUD, S. W. Separation of the various penicillins by partition chromatography i
DODGSON, K. S. & WILLIAMS, R. T. Studies in detoxication. 27. The orientation of conjugation in the metabolites of 4-chlorocatechol and 4-chlororesorcinol, with some observations on the fate of (+)-adrenaline, protocatechuic acid and protocatechuic aldehyde in the rabbit 381
DOLBY, D. E. *see* CHATTAWAY, F. W. 592
DOMINGO, W. R. & KLYNE, W. A photoelectric flame photometer 400
DUVE, C. de *see* NYS, A. 245

EARLY, D. F., HEMPHILL, R. E., REISS, M. & BRUMMEL, E. Investigations into cholinesterase levels in serum and cerebrospinal fluid of psychotic patients 552
EASTHAM, M. Paper-partition chromatography of the sugars in urine xiii
*EASTHAM, M. The chemical changes in carbohydrates during growth of the pea (*Pisum sativum*) xvii
EDELMAN, J. *see also* BACON, J. S. D. xxviii
EDELMAN, J. & BACON, J. S. D. The action of an enzyme preparation from *Helianthus tuberosus* L. (Jerusalem artichoke) on carbohydrates present in the tubers xxix
EDEN, E. & SELLERS, K. C. The absorption of vitamin A xxxiii
EGGLESTON, L. V. & KREBS, H. A. Optical rotation of the molybdate complex of *dextro-iso*citric acid 578
ELLINGER, P. & ABDEL KADER, M. M. Tryptophan and the biosynthesis of nicotinamide 276
ELLIOTT, D. F. Isolation of L-threonine from proteins 429
ELLIOTT, D. F. & NEUBERGER, A. Irreversibility of the deamination of threonine in the rat xiii
EMERY, W. B. *see* LEES, K. A. ii
EVANS, E. T. R. *see* ROBERTS, H. E. xxx
EVANS, R. A. *see* PARR, W. H. xxix
EVANS, W. C. *see* PARR, W. H. xxix; ROBERTS, H. E. xxx

FLEMING, D. S., MOORE, A. M. & BUTLER, G. C. Observations on antisera against complexes of di-2-chloroethyl sulphide and protein 546
*FLOWERS, H. M. & REITH (REICH), W. S. Some remarks on the hydrolysis of proteins xx
FOLLEY, S. J. & FRENCH, T. H. The intermediary metabolism of the mammary gland. 1. Respiration of lactating mammary gland slices in presence of carbohydrates 117
FOLLEY, S. J. & FRENCH, T. H. The intermediary metabolism of the mammary gland. 2. Respiration and acid production of mammary tissue during pregnancy, lactation and involution in the rat 270
FOTHERGILL, P. *see* CHALLENGER, F. xxvii
FREE, A. A. *see* CLAYTON, J. C. xx
FREEMAN, G. G. & MACPHERSON, C. S. Studies on metabolic products of the *Penicillium luteum* series 179
FREEMAN, G. G., MORRISON, R. I. & MICHAEL, S. E. Metabolic products of *Trichothecium roseum* Link 191
FRENCH, T. H. *see* FOLLEY, S. J. 117, 270

GARTON, G. A., ROBINSON, D. & WILLIAMS, R. T. Studies in detoxication. 25. The characterization of phenylglucuronide, and its rate of hydrolysis compared with that of phenylsulphuric acid 65
GARTON, G. A. & WILLIAMS, R. T. Studies in detoxication. 26. The fates of phenol, phenylsulphuric acid and phenylglucuronide in the rabbit, in relation to the metabolism of benzene 158
GILBERT, C. W. *see* DALE, W. M. 93, 543
GOODWIN, T. W. The biochemistry of locusts. 2. Carotenoid distribution in solitary and gregarious phases of the African migratory locust (*Locusta migratoria migratorioides*, R. & F.) and the desert locust (*Schistocerca gregaria* Forsk) 472
GOODWIN, T. W. & SRISUKH, S. Some observations on astaxanthin distribution in marine crustacea 268
GOODWIN, T. W. & SRISUKH, S. The biochemistry of locusts. 1. The carotenoids of the integument of two locust species (*Locusta migratoria migratorioides* R. & F. and *Schistocerca gregaria* Forsk.) 263
GOODWIN, T. W. & WILSON, A. A. Studies in vitamin A. 14. The alleged mobilization of vitamin A by adrenaline 370
GOODWIN, T. W. *see also* CAMA, H. R. 236, 317
GORDON, A. H. An investigation of the intracellular fluid of calf embryo muscle 99
GORDON, J. J. *see* REISS, M. 574
GORNALL, A. G. *see* BARDAWILL, C. J. iii
GRAY, C. H., NEUBERGER, A. & SNEATH, P. H. A. Stercobilin formation in a case of congenital porphyria and in the normal xvi

HALE, J. H. *see* RAWLINSON, W. A. 247
HALL, D. A. *see* CHATTAWAY, F. W. 592
*HAPPOLD, F. C. & MORRISON, G. A. The energy of activation of the tryptophanase-tryptophan indole reaction xx

HAPPOLD, F. C. *see also* CHATTAWAY, F. W. 592
HELLIWELL, P. J. *see* BRAIN, F. H. 75
HEMPHILL, R. E. *see* EARLY, D. F. 552; REISS, M. 574
HERBERT, F. K. Plasma and tissue protein regeneration in renal disease with hypoproteinaemia and oedema xxii
HIGGINBOTTOM, C. *see* DEVI, P. 391
HILLS, G. M. Chemical factors in the germination of spore-bearing aerobes. The effect of yeast extract on the germination of *Bacillus anthracis*, and its replacement by adenosine 353
HILLS, G. M. Chemical factors in the germination of spore-bearing aerobes. The effects of amino-acids on the germination of *Bacillus anthracis*, with some observations on the relation of optical form to biological activity 363
HOCH, H. A differential method for the detection of small differences in mobility of colloids in electrophoresis 285
HOLMAN, W. I. M. & LANGE, D. J. de. Methods for the determination of *N*-methyl-2-pyridone-5-carboxylamide and of *N*-methyl-2-pyridone-3-carboxylamide in human urine 559
HOLT, P. F. & YATES, D. M. Naturally occurring organo-silicon compounds xxxii
HOROWITZ, N. H. *see* BENDER, A. E. xxi
HOWARD, G. A. *see* POLLOCK, M. R. 417
HUGHES, D. E. Acceleration of bacterial glutamic decarboxylase and glutaminase by cetyltrimethylammonium bromide 325
HUGHES, D. E. A modified chemical estimation of nicotinic acid xxxiv
HUGHES, D. E. & WILLIAMSON, D. H. Cozymase synthesis by *Lactobacillus arabinosus* 17–5 xxxvi
HUMPHRIS, B. G. *see* BRAY, H. G. 241
HUTCHINSON, A. O. *see* MCCANCE, R. A. 493

JACKSON, E. M. *see* ROOKE, H. S. 231
JAMES, S. P. *see* BRAY, H. G. 467
JOHNSON, A. C., MCNABB, A. R. & ROSSITER, R. J. Chemical studies of peripheral nerve during Wallerian degeneration. 1. Lipids 500
JONES, P. E. H. & MCCANCE, R. A. Enzyme activities in the blood of infants and adults 464
JONES, P. E. H. *see also* MCCANCE, R. A. 493
JONES, R. L., METCALFE, T. P. & SEXTON, W. A. The relationship between the constitution and the effect of chemical compounds on plant growth. 1. 2-Phenoxyethylamine derivatives 143
JUDAH, J. H. The micro- and ultramicro-determination of chlorine in organic compounds 60

KARUNAIRATNAM, M. C., KERR, L. M. H. & LEVVY, G. A. The glucuronide-synthesizing system in the mouse and its relationship to β-glucuronidase 496
KEILIN, J. The reactions of haems with cyanides and *iso*-cyanides 440
KEILIN, J. On the properties and nature of dihydroxyl-haem 448
KENTEN, R. H. & MANN, P. J. G. The oxidation of manganese by plant extracts in the presence of hydrogen peroxide 255
KENTEN, R. H. *see also* BOWES, J. H. 281
KERR, L. M. H. *see* KARUNAIRATNAM, M. C. 496
KING, E. J. *see* ABUL-FADL, M. A. M. 51
KLYNE, W. The configuration at C-20 of the urinary pregnane-3(α):17(α):20-triol of Butler and Marrian viii
KLYNE, W. *see also* DOMINGO, W. R. 400
KODICEK, E. & MISTRY, S. P. An improved medium for microbiological assays with *Lactobacillus casei* xxxv
KON, S. K. & THOMSON, S. Y. Preformed vitamin A in northern krill xxxi
KREBS, H. A. *see* BENDER, A. E. xxi; EGGLESTON, L. V. 578

LAMPITT, L. H. *see* ROOKE, H. S. 231
LANGE, D. J. de *see* HOLMAN, W. I. M. 559
LASER, H. & ROTHSCHILD, Lord. A new manometric method for determination of respiratory quotients 598
LAWRIE, N. R. & YUDKIN, J. Studies in biochemical adaption. Effect on diet on the intestinal phosphatase of the rat. 438
LEES, K. A. & EMERY, W. B. Microbiological assay of growth factors essential for *Lactobacillus leichmanii* 313 and *Leuconostoc citrovorum* ii
LESLIE, I. *see* DAVIDSON, J. N. xv
LESTER SMITH, E. & CUTHBERTSON, W. F. J. Paper chromatography of the vitamin B_{12} group of factors xii
LEVVY, G. A. *see* KARUNAIRATNAM, M. C. 496
LIÉBECQ, C. *see* COXON, R. V. 320
*LLOYD, J. T. *see* CUTHBERTSON, W. F. J. xvii
LONG, C. The respiration of rat-liver slices in the presence of some aliphatic amines, hydroxy-amines and quaternary ammonium salts xi
LOWTHER, A. G. & REITH (REICH), W. S. Separation of *N*-2:4-dinitrophenyl derivatives of the methyl esters of amino-acids by adsorption chromatography vi

MCCANCE, R. A., HUTCHINSON, A. O., DEAN, R. F. A. & JONES, P. E. H. The cholinesterase activity of the serum of newborn animals and of colostrum 493
MCCANCE, R. A. *see also* JONES, P. E. H. 464
MCCLYMONT, G. L. Acetic acid in bovine peripheral blood and its utilization by the mammary gland i
MCDOUGALL, E. I. The composition of foetal fluids of sheep at different stages of gestation 397
MCILWAIN, H. & RODNIGHT, R. Breakdown of the oxidized forms of coenzymes I and II by an enzyme from the central nervous system 337
MCINDOE, W. M. *see* DAVIDSON, J. N. xvi
MCNABB, A. R. *see* JOHNSON, A. C. 500
MCNAUGHT, M. L. *see* CHANDA, R. xix
MACPHERSON, C. S. *see* FREEMAN, G. G. 179
MALPRESS, F. H. & MORRISON, A. B. The semi-micro estimation of lactose alone and in the presence of other sugars 455
MANN, P. J. G. *see* KENTEN, R. H. 255
MAPSON, L. W., CRUICKSHANK, E. M. & CHEN, YU-TUAN. Factors affecting synthesis of ascorbic acid in cress seedlings. 2. Ascorbic acid synthesis in relation to sugar formation 171
MARKHAM, R. & SMITH, J. D. Chromatographic studies of nucleic acids. 1. A technique for the identification and estimation of purine and pyrimidine bases, nucleosides and related substances 294
MARKHAM, R. & SMITH, J. D. The quantitative analysis of ribonucleic acids xxxii
MARRIAN, D. H., RUSSELL, P. B. & TODD, A. R. The potato eelworm hatching factor. 6. Attempts to prepare artificial hatching agents. Part II. Some active arylidene-$\Delta^{\beta:\gamma}$-butenolides and related compounds 533
MARRIAN, D. H., RUSSELL, P. B., TODD, A. R. & WARING, W. S. The potato eelworm hatching factor. 3. Concentration of the factor by chromatography. Observations on the nature of eclepic acid 524
MELVILLE, R. *see* WOKES, F. 343
METCALFE, T. P. *see* JONES, R. L. 143
MICHAEL, S. E. *see* FREEMAN, G. G. 191
MISTRY, S. P. *see* KODICEK, E. xxxv
MOORE, A. M. *see* FLEMING, D. S. 546
MORRISON, A. B. *see* MALPRESS, F. H. 455
MORRISON, G. A. *see* DAGLEY, S. xxv, xxvi; HAPPOLD, F. C. xx*
MORRISON, R. I. *see* FREEMAN, G. G. 191
MORTON, R. A. & ROSEN, D. G. Carotenoids, vitamin A and 7-dehydrosteroid in the frog (*Rana temporaria*) 612

MORTON, R. A. *see also* BALL, S. 298, 304; BARUA, R.K. 308
MOYLE, J. M. *see* STEPHENSON, M. vii
MUIR, H. M. & NEUBERGER, A. Further studies of the biogenesis of porphyrins in rabbits xxxiv
MUIR, H. M. & NEUBERGER, A. The biogenesis of porphyrins. The distribution of ^{15}N in the ring system 163
MUIR, H. M. *see also* POPJÁK, G. xi

NEUBERGER, A. *see* ARNSTEIN, H. R. V. iii; BENTLEY, R. 584; ELLIOTT, D. F. xiii; GRAY, C. H. xvi; MUIR, H. M. 163, xxxiv; NIVEN, J. S. F. iv
NICHOLAS, R. E. H. & COMFORT, A. Acid-soluble pigments of molluscan shells. 4. Identification of shell porphyrins with particular reference to conchoporphyrin 208
NICHOLSON, T. F. Renal functions as affected by experimental unilateral kidney lesions. 2. The effect of cyanide 112
NIVEN, J. S. F. & NEUBERGER, A. Studies on porphyrin metabolism in the rabbit iv
NYS, A., AUBERT, X. & DUVE, C. de. The distribution of glycogen in the liver of rabbits 245

OGSTON, A. G. The sedimentation and diffusion of polysaccharides from *Penicillium luteum*; interpretation of the results obtained from polydisperse material in the Gouy diffusiometer 189
OLLEY, J. *see* CAMPBELL, I. G. 105
O'MEARA, R. A. Q. *see* BELL, E. A. 373
OWEN, E. C. *see* CHANDA, R. xix

PAGE, J. E. *see* CLAYTON, J. C. xx
PALMER, A. *see* BELL, D. J. xiv
PARR, W. H., EVANS, R. A. & EVANS, W. C. Themechanism of the bacterial oxidation of certain aromatic compounds, together with the preparation and properties of a cell-free enzyme system which accomplishes ring cleavage xxix
PARTRIDGE, S. M. Displacement chromatography on synthetic ion-exchange resins. 4. The isolation of glucosamine and histidine from a protein hydrolysate 459
PETERS, R. A. *see* COXON, R. V. 320
POLLOCK, M. R., HOWARD, G. A. & BOUGHTON, B. W. Long-chain unsaturated fatty acids as essential bacterial growth factors. Substances able to replace oleic acid for the growth of *Corynebacterium* 'Q' with a note on a possible method for their microbiological assay 417
PORTER, H. K. The inhibition of plant phosphorylases by β-amylase and the detection of phosphorylase in barley xxxvii
POPJÁK, G. & BEECKMANS, M.-L. Are phospholipids transmitted through the placenta? x
POPJÁK, G. & MUIR, H. M. In search of a P-precursor of phospholipids xi

RAISTRICK, H. *see* CALAM, C. T. 513
RAMSAY, W. N. M. Acid-soluble phosphorus in hen eggs during development of the embryo ix
RAMSAY, W. N. M. Iron metabolism in hen eggs during development of the embryo viii
RAWLINSON, W. A. & HALE, J. H. Prosthetic groups of the cytochromes present in *Corynebacterium diphtheriae* with especial reference to cytochrome *a* 247
REISS, M., HEMPHILL, R. E., GORDON, J. J. & COOK, E. R. Regulation of urinary steroid excretion. 2. Spontaneous changes in the pattern of daily excretion in mental patients 574
REISS, M. *see also* EARLY, D. F. 552
REITH (REICH), W. S. *see* FLOWERS, H. M. xx; LOWTHER, A. G. vi
ROBERTS, E. A. H. The fermentation process in tea manufacture. 10. The condensation of catechins and its relation to the chemical changes in fermentation 538
ROBERTS, H. E., EVANS, E. T. R. & EVANS, W. C. Some observations on the so-called "bracken poisoning' of farm animals, and the aneurin inactivating mechanism of various plants xxx
ROBINSON, D. *see* GARTON, G. A. 65
ROBINSON, J. R. Some effects of glucose and calcium upon the metabolism of kidney slices from adult and newborn rats 68
RODAHL, K. & DAVIES, A. W. Vitamin A in seals 408
RODNIGHT, R. *see* MCILWAIN, H. 337
ROGERS, H. J. The metabolism of the amino sugars. 1. The breakdown of *N*-acetylglucosamine by strains of *Streptococcus haemolyticus* and other streptococci 87
ROGERS, H. J. Observations on the organic matrix of bone xxiv
ROGERS, H. J. *see also* WEIDMANN, S. xxv
ROOKE, H. S., LAMPITT, L. H. & JACKSON, E. M. The phosphorus compounds of wheat starch 231
ROSEN, D. G. *see* MORTON, R. A. 612
ROSSITER, R. J. *see* JOHNSON, A. C. 500
ROTHSCHILD LORD *see* LASER, H. 598
RUSSELL, P. B., TODD, A. R. & WARING, W. S. The potato eelworm hatching factor. 4. *Solanum nigrum* as a source of the potato eelworm hatching factor 528
RUSSELL, P. B., TODD, A. R. & WARING, W. S. The potato eelworm hatching factor. 5. Attempts to prepare artificial hatching agents. Part I. Some furan derivatives 530
RUSSELL, P. B. *see also* MARRIAN, D. H. 524, 533

SANGER, F. The terminal peptides of insulin 563
SELLERS, K. C. *see* EDEN, E. xxxiii
SEXTON, W. A. *see* JONES, R. L. 143
SLACK, E. B. *see* CHICK, H. 211
SLATER, E. C. A comparative study of the succinic dehydrogenase-cytochrome system in heart muscle and in kidney 1
SLATER, E. C. A respiratory catalyst required for the reduction of cytochrome *c* by cytochrome *b* 14
SLATER, E. C. Effect of sulphydryl-combining compounds on the activity of the succinic oxidase system 130
SLATER, E. C. The action of inhibitors on the system of enzymes which catalyse the aerobic oxidation of succinate 8
SLOANE-STANLEY, G. H. Amino-acid decarboxylase of rat liver 556
SMITH, J. D. *see* MARKHAM, R. 294, xxxii
SMITH, J. N. Studies in detoxication. 28. The biosynthesis of *o*, *m*- and *p*-cyanophenylglucuronides 638
SNEATH, P. H. A. *see* GRAY, C. H. xvi
SOMERS, G. F. *see* CLAYTON, J. C. xx
SRISUKH, S. *see* GOODWIN, T. W. 263, 268
STEPHENSON, M. & MOYLE, J. M. Nucleic acid metabolism of *Escherichia coli* vii
STICKLAND, L. H. Some observations on the coenzyme of phosphoglucomutase xxi
STROUD, S. W. *see* DOBSON, F. i

TÁRNOKY, A. L. & BREWS, V. A. L. Determination of *p*-aminosalicylic acid as *m*-aminophenol 508
TERNER, C. The reduction of gastric acidity by back-diffusion of hydrogen ions through the mucosa 150
THOMPSON, R. H. S. & TICKNER, A. Observations on the mono-amine oxidase activity of placenta and uterus 125
THOMPSON, S. Y. *see* KON, S. K. xxxi
THOMSON, R. O. The distribution of lipoids in the faeces of normal and cholesterol-fed animals xiv
THORPE, W. V. *see* BRAY, H. G. 45, 241, 467
TICKNER, A. *see* THOMPSON, R. H. S. 125
TODD, A. R. *see* CALAM, C. T. 513, 530; MARRIAN, D. H. 524, 533; RUSSELL, P. B. 528, 530

TODD, C. M. Occurence of cytochrome and coproporphyrin in mycobacteria 386
TSAO, T. C. *see* ADAIR, G. S. v

WARING, W. S. *see* CALAM, C. T. 520; MARRIAN, D. H. 524; RUSSELL, P. B. 528, 530
WASDELL, M. R. *see* BRAY, H. G. 467
WEIDMANN, S. & ROGERS, H. J. Preliminary observations on the metabolism of the alveolar bone of mammals xxv
WEIL-MALHERBE, H. & BONE, A. D. The micro-estimation of citric acid 377
WILLIAMS, R. T. *see* DODGSON, K. S. 381; GARTON, G. A. 65, 158
WILLIAMSON, D. H. *see* HUGHES, D. E. xxxvi
WILSON, A. A. *see* GOODWIN, T. W. 370
WILSON, E. J. & WORMALL, A. Studies on suramin (Antrypol: Bayer 205). 7. Further observations on the combination of the drug with proteins 224
WOIWOD, A. J. A method for the estimation of micro amounts of amino nitrogen and its application to paper partition chromatography 412
WOKES, F. & MELVILLE, R. Apparent vitamin C in the walnut (*Juglans regia*) 343
WOOD, P. B. *see* BRAY, H. G. 45, 467
WOOLLETT, E. A. *see* CLAYTON, J. C. xx
WORMALL, A. *see* WILSON, E. J. 224

YATES, D. M. *see* HOLT, P. F. xxxii
YUDKIN, J. *see* LAWRIE, N. R. 438

ZILVA, S. S. Vitamin P. 3 79

Index of Subjects

Note. Entries marked with an asterisk (*) refer to communications made in title only.
Entries marked with a section mark (§) refer to contributions to symposia.
Small roman numerals refer to pages in *Proceedings of the Biochemical Society.*

Abalone, pigments of (Comfort, A.) 204
Absorption apparatus for trichloroethylene distilled in steam (Brain, F. H. & Helliwell, P. J.) 92
Absorption of DL-thyroxine labelled with radioactive iodine (Clayton, J. C., Free, A. A., Page, J. E., Somers, G. J. & Woolett, E. A.) xx
Absorption of vitamin A (Eden, E. & Sellers, K. C.) xxxiii
Absorption, ultraviolet, of phenylglucuronidic acid (Garton, G. A., Robinson, D. & Williams, R. T.) 65
Acetamide and acetic acid hydrolysis by rabbit-liver preparation (Bray, H. G., James, S. P., Thorpe, W. V., Wasdell, M. R. & Wood, P. B.) 467
Acetamide and acetic acid metabolism in rabbits (Bray, H. G., James, S. P., Thorpe, W. V., Wasdell, M. R. & Wood, P. B.) 467
Acetate, conversion to glycine in rat (Arnstein, H. R. V. & Neuberger, A.) iii
Acetate formation by *Clostridium saccharobutyricum* (Cohen-Bazire, G. & Cohen, G. N.) 41
Acetate, formation by oxidation of pyruvate by pigeon brain (Coxon, R. V., Liébecq, C. & Peters, R. A.) 320
Acetic acid in bovine peripheral blood, utilization by the mammary gland (McClymont, G. L.) i
N-Acetylglucosamine, breakdown by strains of streptococci (Rogers, H. J.) 87
Acid phosphatase activity in eythrocytes of infants and adults (Jones, P. E. H. & McCance, R. A.) 464
Acid phosphatases of erythrocytes and of human prostate gland (Abul-Fadl, M. A. M. & King, E. J.) 51
Acid production of mammary tissue during pregnancy, lactation and involution in the rat (Folley, S. J. & French, T. H.) 270
Acidity, gastric, reduction by back-diffusion of hydrogen ions through the mucosa (Terner, C.) 150
Acids, fatty, long-chain, unsaturated, as essential bacterial growth factors (Pollock, M. R., Howard, G. A. S. & Boughton, B. W.) 417
Acid-soluble phosphorus in hen eggs during development of embryo (Ramsay, W. N. M.) ix
Activation, energy of, of the tryptophanase-tryptophan indole reaction (Happold, F. C. & Morrison, G. A.) xx
Adaptation, biochemical, effect of diet on intestinal phosphatases of rat (Lawrie, N. R. & Yudkin, J.) 438
Adenosine, effect on germination of *Bacillus anthracis* (Hills, G. M.) 253
Adenosinediphosphate, enzyme assay of (Bailey, K.) 479
Adenosinediphosphate, formation from adenosinetriphosphate on storage (Bailey, K.) 479
Adenosinetriphosphate, enzymic degradation (Bailey, K.) 479
Adenylic acid formation from adenosinetriphosphate on storage (Bailey, K.) 479
Adrenaline, alleged mobilization of vitamin A by (Goodwin, T. W. & Wilson, A. A.) 370
(+)-Adrenaline, fate in rabbit (Dodgson, K. S. & Williams, R. T.) 381
Adrenaline, inactivation of serum alkaline phosphatase by (Anderson, A. B.) xxxvi
Adsorption chromatography, separation of *N*-2:4-dinitrophenyl derivatives of methyl esters of amino-acids by (Lowther, A. G. & Reith (Reich), W. S.) vi
Adults, enzyme activities of blood of (Jones, P. E. H. & McCance, R. A.) 464
Aerobacter aerogenes, 'early-lag' phase of (Dagley, S., Dawes, A. E. & Morrison, G. A.) xxv
Aerobacter aerogenes, mutants of, requiring nicotinic acid and histidine (Devi, P. & Higginbottom, C.) 391
Aerobes, spore-bearing, germination of (Hills, G. M.) 353, 363
Aerobic oxidation of succinate, inhibitors of (Slater, E. C.) 8
Alanine, effect on germination of spores of *Bacillus anthracis* (Hills, G. M.) 363
Albumins, urine and serum, mobility in electrophoresis (Hoch, H.) 285
Alkaline haemochromogen (Keilin, J.) 448
Alkaloid salt fractionation for purification of potato eelworm hatching factor (Calam, C. T., Todd, A. R. & Waring, W. S.) 520
Allantoic fluid from ewes fed during pregnancy, composition of during gestation (McDougall, E. I.) 397
Alveolar bone, metabolism of, in mammals (Weidmann, S. & Rogers, H. J.) xxv
Amide nitrogen of substances from connective tissue (Bowes, J. H. & Kenten, R. H.) 281
Amides, lower aliphatic, fate in rabbit (Bray, H. G., James, S. P., Thorpe, W. V., Wasdell, M. R. & Wood, P. B.) 467
Amides of potato juice (Chick, H. & Slack, E. B.) 211
Amides of toluic acids, fate in rabbit (Bray, H. G., Thorpe, W. V. & Wood, P. B.) 45
Amines, aliphatic, respiration of rat-liver slices in presence of (Long, C.) xi
Amino compounds, reactions with retinene, (Ball, S., Collins, F. D., Dalvi, P. D. & Morton, R. A.) 304
Amino nitrogen, estimation of micro amounts and its application to paper-partition chromatography (Woiwod, A. J.) 412
Amino sugars, metabolism of (Rogers, H. J.) 87
Amino-acid decarboxylases of rat liver (Sloane-Stanley, G. H.) 556
Amino-acid distribution of collagen, elastin and reticular tissue from different sources (Bowes, J. H. & Kenten, R. H.) 281
Amino-acids, deamination by X-rays (Dale, W. M., Davies, J. V. & Gilbert, C. W.) 93
Amino-acids, *N*-2:4-dinitrophenyl derivatives of methyl esters of, separation by adsorption chromatography (Lowther, A. G. & Reith (Reich), W. S.) vi
Amino-acids, *N*-2:4-dinitrophenyl, use of buffered columns in the chromatographic separation of (Blackburn, S.) 579
Amino-acids, effect on germination of *Bacillus anthracis* (Hills, G. M.) 363
Amino-acids, hydroxy, separation from proteins and hydrolysates (Elliott, D. F.) 429
Amino-acids in nisin (Berridge, N. J.) 486
Amino-acids, influence on 'early lag' (Dagley, S., Dawes, E. A. & Morrison, G. A.) xxvi
Amino-acids of potato juice (Chick, H. & Slack, E. B.) 211
Amino-acid oxidase of *Neurospora crassa* (Bender, A. E., Krebs, H. A. & Horowitz, N. H.) xxi
Amino-acids, separation by ion-exchange resins (Davies, C. W.) 38
p-Aminobenzoic acid, condensation products with reductone (Bell, E. A., Cocker, W. & O'Meara, R. A. Q.) 373
4-Amino-2-hydroxybenzoic acid *see p*-Aminosalicylic acid
m-Aminophenol, determination of *p*-aminosalicylic acid as (Tárnoky, A. L. & Brews, V. A. L.) 508

p-Aminophenylarsenoxide, effect on succinic dehydrogenase and succinic oxidase systems. (Slater, E. C.) 130
p-Aminosalicylic acid, determination as *m*-aminophenol (Tárnoky, A. L. & Brews, V. A. L.) 508
Ammonia, formation from *N*-acetylglucosamine by streptococci (Rogers, H. J.) 87
Ammonia, liberation from glycine by X-rays (Dale, W. M., Davies, J. V. & Gilbert, C. W.) 543
Ammonium salts, quaternary, respiration of rat-liver slices in presence of (Long, C.) xi
Amniotic fluid from ewes fed during pregnancy, composition of (McDougall, E. I.) 397
β-Amylase, inhibition of plant phosphorylases by (Porter, H. K.) xxxvii
Aneurin deficiency and interrelationship of vitamins B (Bhagvat, K. & Devi, P.) 32
Aneurin-inactivating mechanism of plants (Roberts, H. E., Evans, E. T. R. & Evans, W. C.) xxx
Anhydrotetronic acid as an artificial hatching agent for potato eelworm (Calham, C. T., Todd, A. R. & Waring, W. S.) 520
Anterior pituitary hormones and mammary gland metabolism of (Folley, S. J. & French, T. H.) 270
Antibiotics, preparation of nisin (Berridge, N. J.) 486
Antisera against complexes of di-2-chloroethyl sulphide and protein (Fleming, D. S., Moore, A. M. & Butler, G. C.) 546
Antrypol *see* Suramin
Apparatus for micro-electrolysis of water (Bentley, R.) 591
Apyrase, potato, use for assay of adenosinetriphosphate (Bailey, K.) 479
Archaeogastropods, pigments of (Comfort, A.) 199
Aromatic compounds, bacterial oxidation of (Parr, W. H., Evans, R. A. & Evans, W. C.) xxix
Artichoke, Jerusalem, enzyme preparation from, action on carbohydrates present in the tubers of (Edelman, J. & Bacon, J. S. D.) xxix
Arylidene-$\Delta^{\beta\cdot\gamma}$-butenolides as artificial hatching agents for potato eelworm (Marrian, D. H., Russell, P. B. & Todd, A. R.) 533
Ascorbic acid distribution in developing salmon (Daniel, R. J.) 435
Ascorbic acid, factors affecting synthesis in cress seedlings (Mapson, L. W., Cruickshank, E. M. & Chen, Yu-Tuan) 171
Assay, microbiological, of substances able to replace oleic acid as growth factor of *Corynebacterium* 'Q' (Pollock, M. R., Howard, G. A. & Boughton, B. W.) 417
Astaxanthin distribution in locusts in solitary and gregarious phases (Goodwin, T. W.) 472
Astaxanthin distribution in marine crustacea (Goodwin, T. W. Srisukh, S.) 268
Astaxanthin in locusts (Goodwin, T. W. & Srisukh, S.) 263

Bacillus anthracis, effect of amino-acids on germination of (Hills, G. M.) 363
Bacillus anthracis, germination of, effect of yeast extract and of adenosine on (Hills, G. M.) 353
Back-diffusion of hydrogen ions through the mucosa, reduction of gastric acidity by (Terner, C.) 150
Bacterial growth factors, essential, long-chain unsaturated fatty acids as (Pollock, M. R., Howard, G. A. & Boughton B. W.) 417
Bacterial oxidation of aromatic compounds mechanism of (Parr, W. H., Evans, R. A. & Evans, W. C.) xxix
Bacterium aerogenes, mutants of, requiring nicotinic acid and histidine (Devi, P. & Higginbottom, C.) 391
BAL *see* 2:3-Dimercaptopropanol
Barley, detection of phosphorylase in (Porter, H. K.) xxxvii
Bayer 205 *see* Suramin
Benzene metabolism in rabbit (Garton, G. A. & Williams, R. T.) 158
Benziminoethyl ether, use in isolation of threonine and serine from proteins (Elliott, D. F.) 429
Bile pigments, possible relationship of *Haliotis* pigments to (Comfort, A.) 204
Bile salts, effect on succinic oxidase activity (Slater, E.C.) 8
Bioassay method for the hatching factor of potato eelworm cysts (Calam, C. T., Raistrick, H. & Todd, A. R.) 513
Biogenesis of porphyrins (Muir, H. M. & Neuberger, A.) 163, xxxiv
Biological activity of *Bacillus anthracis* relation to optical form (Hills, G. M.) 363
Biosynthesis of *o*-, *m*- and *p*-cyanophenylglucuronides (Smith, J. N.) 638
Biotin deficiency, interrelationship of vitamins B (Bhagvat, K. & Devi, P.) 32
Bivalves, pigments of (Comfort, A.) 199
Blastoderm of salmon, ascorbic acid in (Daniel, R. J.) 435
Blood, bovine peripheral, acetic acid in (McClymont, G. L.) i
Blood, determination of sodium and potassium in by flame photometer (Domingo, W. R. & Klyne, W.) 400
Blood, estimation of 'total *p*-aminosalicylate' in (Tárnoky, A. L. & Brews, V. A. L.) 508
Blood, estimation of trichloroethylene in (Brain, F. H. & Helliwell, P. J.) 75
Blood of infants and adults, enzyme activities of (Jones, P. E. H. & McCance, R. A.) 464
Blood of locusts, β-carotene in (Goodwin, T. W. & Srisukh, S.) 263
Blubber of seals (Rodahl, K. & Davies, A. W.) 408
Body size and tissue activity (Folley, S. J. & French, T. H.) 117
Bone, alveolar, metabolism in mammals (Weidmann, S. & Rogers, H. J.) xxv
Bone, organic matrix of (Rogers, H. J.) xxiv
Boron, α-radiation from disintegration of, in a nuclear reactor (Dale, W. M., Davies, J. W. & Gilbert, C. W.) 543
Boundaries in electrophoresis (Hoch, H.) 285
Bovine peripheral blood, acetic acid in (McClymont, G. L.) i
Bracken poisoning of farm animals (Roberts, H. E., Evans, E. T. R., & Evans W. C.) xxx
Brain, pyruvate-oxidase system in (Coxon, R. V., Liébecq, C. & Peters, R. A.) 320
Brassidic acid and growth of *Corynebacterium* 'Q' (Pollock, M. R., Howard, G. A. & Boughton, B. W.) 417
Brucine salt of eclepic acid (Calam, C. T., Todd, A. R. & Waring, W. S.) 520
Buds of walnuts, apparent vitamin C in (Wokes, F. & Melville, R.) 343
Buffered columns, use in chromatographic separation of 2:4-dinitrophenyl amino-acids (Blackburn, S.) 579
Butanolides and hatching of potato eelworm (Russell, P. B., Todd, A. R. & Waring, W. S.) 530
Butenolides and hatching of potato eelworm (Russell, P. B., Todd, A. R. & Waring, W. S.) 530
Butenolides, arylidene-$\Delta^{\beta\cdot\gamma}$-, and hatching of potato eelworm (Marrian, D. H., Russell, P. B. & Todd, A. R.) 533
n-Butyramide and butyric acid, hydrolysis by rabbit-liver preparation (Bray, H. G., James, S. P., Thorpe, W. V., Wasdell, M. R. & Wood, P. B.) 467
n-Butyramide and butyric acid metabolism in rabbits (Bray, H. G., James, S. P., Thorpe, W. V., Wasdell, M. R. & Wood, P. B.) 467
Butyrate formation by *Clostridium saccharobutyricum* (Cohen-Bazire, G. & Cohen, G. N.) 41
Byssochlamys fulva, disaggregating enzyme of (Beaven, G. H., & Brown F.) 221
Byssochlamys fulva, pectic enzmyes of (Beaven, G. H. & Brown, F.) 221
Byssochlamys fulva, protopectinase of (Beaven, G. H. & Brown, F.) 221

Calcium, effect on the metabolism of kidney slices from adult and newborn rats (Robinson, J. R.) 68
Calcium in foetal fluids of sheep during gestation (McDougall, E. I.) 397
Calcium phosphates, solubility of (Robinson, J. R.) 68
Calf embryo muscle, intracellular fluid of (Gordon, A. H.) 99
Capillary resistance of guinea pigs (Zilva, S. S.) 79
Carapaces of lobster and prawn, astaxanthin in (Goodwin, T. W. & Srisukh, S.) 268
Carbohydrates, chemical changes during growth of the pea (Eastham, M.) xvii
Carbohydrates, effect on respiration of lactating mammary gland slices (Folley, S. J. & French, T. H.) 117
Carbohydrates in subterranean organs of some Compositae (Bacon, J. S. D. & Edelman, J.) xxviii
Carbohydrates of tubers, action of an enzyme preparation from *Helianthus tuberosus* L. (Jerusalem artichoke) on (Edelman, J. & Bacon, J. S. D.) xxix
Carbon dioxide production, manometric method for determination (Laser, H. & Rothschild, Lord) 598
Carbon monoxide-carbylamine-haem compounds (Keilin, J.) 440
Carbon monoxide-cyan-haem compounds (Keilin, J.) 440
Carbonic anhydrase, activity in erythrocytes of infants and adults (Jones, P. E. H. & McCance, R. A.) 464
Carbylamine-haem compounds, formation by combination of methyl *iso*cyanide and haems (Keilin, J.) 440
β-Carotene, alleged formation of vitamin A from (Cama, H. R. & Goodwin, T. W.) 317
β-Carotene, distribution in locusts in solitary and gregarious phases (Goodwin, T. W.) 472
β-Carotene in locusts (Goodwin, T. W. & Srisukh, S.) 263
Carotene, role of the thyroid in metabolism of (Cama, H. R. & Goodwin, T. W.) 236
Carotenoid distribution of African migratory locusts and desert locusts (Goodwin, T. W.) 472
Carotenoids in the frog, analytical methods of determination (Morton, R. A. & Rosen, D. G.) 612
Carotenoids of the integument of locusts, (Goodwin, T. W. & Srisukh, S.) 263
Carrot (*Daucus carota*), glutamic decarboxylase of (Hughes, D. E.) 325
Casein, iodinated, alleged formation of vitamin A from β-carotene treated with (Cama, H. R. & Goodwin, T. W.) 317
Catalase activity in erythrocytes of infants and adults (Jones, P. E. H. & McCance, R. A.) 464
Catalyst, respiratory, required for the reduction of cytochrome *c* by cytochrome *b* (Slater, E. C.) 14
Catechins, condensation of and relation to chemical changes in tea fermentation (Roberts, E. A. H.) 538
Cations, interference in flame photometer (Domingo, W. R. & Klyne, W.) 400
Catkins of walnut, apparent vitamin C in (Wokes, F. & Melville, R.) 343
Cell nucleus, phosphorus compounds in (Davidson, J. N. & McIndoe, W. M.) xvi
Central nervous system, degeneration in, and vitamin A deficiencies in rats (Coetzee, W. A. K.) 628
Central nervous system, degradation of coenzymes I and II by an enzyme from (McIlwain, H. & Rodnight, R.) 337
Cerebroside determination in degenerating cat nerves (Johnson, A. C., McNabb, A. R. & Rossiter, R. J.) 500
Cerebrospinal fluid of psychotic patients, cholinesterase in (Early, D. F., Hemphill, R. E., Reiss, M. & Brummel, E.) 552
Cetavlon *see* Cetyltrimethylammonium bromide (Hughes, D. E.) 325
Cetyltrimethylammonium bromide, acceleration of bacterial glutamic decarboxylase and glutaminase by (Hughes, D. E.) 325
Chick-heart explants grown *in vitro*, chemical composition of (Davidson, J. N. & Leslie, I.) xv
Chloride in foetal fluids of sheep during gestation (McDougall, E. I.) 397
Chlorine, micro- and ultramicro-determination in organic compounds (Judah, J. D.) 60
4-Chlorocatechol metabolites, orientation of conjugation of (Dodgson, K. S. & Williams, R. T.) 381
4-Chloro-2-hydroxyphenylglucuronide from rabbit urine (Dodgson, K. S. & Williams, R. T.) 381
4-Chloro-3-hydroxyphenylglucuronide from rabbit urine (Dodgson, K. S. & Williams, R. T.) 381
p-Chloromercuribenzoate, effect on succinic dehydrogenase and succinic oxidase systems (Slater, E. C.) 130
4-Chlororesorcinol metabolites, orientation of conjugation of (Dodgson, K. S. & Williams, R. T.) 381
Cholesterol determination in degenerating cat nerves (Johnson, A. C., McNabb, A. R. & Rossiter, R. J.) 500
Cholesterol, distribution of lipids in faeces of animals after feeding (Thomson, R. O.) xiv
Choline as phosphatide in wheat starch (Rooke, H. S., Lampitt, L. H. & Jackson, E. M.) 231
Cholinesterase activity of serum of newborn animals and of colostrum (McCance, R. A., Hutchinson, A. O., Dean, R. F. A. & Jones, P. E. H.) 493
Cholinesterase of serum and cerebrospinal fluid of psychotic patients (Early, D. F., Hemphill, R. E., Reiss, M. & Brummel, E.) 552
Cholinesterase of sera and erythrocytes of infant and adult blood (Jones, P. E. A. & McCance, R. A.) 464
Chromatographic separation of 2:4-dinitrophenyl amino-acids, use of buffered columns in (Blackburn, S.) 579
Chromatography, adsorption, separation of *N*-2:4-dinitrophenyl derivative of methyl esters of amino-acids by (Lowther, A. G. & Reith (Reich), W. S.) vi
Chromatography, displacement, on synthetic ion-exchange resins (Partridge, S. M.) 459
Chromatography of nucleic acids (Markham, R. & Smith, J. D.) 294
Chromatography of shell pigments (Comfort, A.) 199
Chromatography, paper, of the vitamin B_{12} group of factors (Lester Smith, E. & Cuthbertson, W. F. J.) xii
Chromatography, paper partition, application of estimation of microamounts of amino nitrogen (Woiwod, A. J.) 412
Chromatography, paper partition, of sugars in urine (Eastham, M.) xiii
Chromatography, partition, separation of penicillins by (Dobson, F. & Strand, S. W.) i
Chromatography, use for purifying potato eelworm hatching factor (Marrian, D. H., Russell, P. B., Todd, A. R. & Waring, W. S.) 524
Chromoproteins as pigments of higher molluscan shells (Comfort, A.) 199
Citrate, formation during oxidation of pyruvate by pigeon brain (Coxon, R. V., Liébecq, C. & Peters, R. A.) 320
Citrate, formation of manganese complex, by hydrogen peroxide and plant extracts (Kenten, R. H. & Mann, P. J. G.) 255
Citric acid, micro-estimation of (Weil-Malberbe, H. & Bone, A. D.) 377
*dextro-iso*Citric acid, optical rotation of molybdate complex of (Eggleston, L. V. & Krebs, H. A.) 578
Cleavage of rings by a cell-free enzyme system (Parr, W. H., Evans, R. A. & Evans, W. C.) xxix
Clostridium saccharobutyricum, Strain GR4, transformation of fumarate to oxaloacetate by (Cohen-Bazire, G. & Cohen, G. N.) 41
Clostridium welchii, glutamic decarboxylase and glutaminase from (Hughes, D. E.) 325

Coenzyme II, inactivation by central nervous system preparations (McIlwain, H. & Rodnight, R.) 337
Coenzyme of phosphoglucomutase (Stickland, L. H.) xxi
Coenzymes I and II, degradation by an enzyme from the central nervous system (McIlwain, H. & Rodnight, R.) 337
Collagen, amino-acid distribution of (Bowes, J. H. & Kenten, R. H.) 281
Collagen, preparation from ox hide (Bowes, J. H. & Kenten, R. H.) 281
Colloids, mobility in electrophoresis, differential method for detection of small differences (Hoch, H.) 285
Colostrum, cholinesterase activity of (McCance, R. A., Hutchinson, A. O., Dean, R. F. A. & Jones, P. E. H.) 493
Columns, buffered, use in the chromatographic separation of 2:4-dinitrophenyl amino-acids (Blackburn, S.) 579
Compositae, carbohydrates present in subterranean organs of (Bacon, J. S. D. & Edelman, J.) xxviii
Conchoporphyrin, identification of (Nicholas, R. E. H. & Comfort, A.) 208
Condensation index of teas during fermentation (Roberts, E. A. H.) 542
Congenital porphyria, stercobilin formation in (Gray, C. H., Neuberger, A. & Sneath, P. H. A.) xvi
Conjugated phenol, fate in rabbit (Garton, G. A. & Williams, R. T.) 158
Conjugation of toluic acids and amides in rabbit (Bray, H. G., Thorpe, W. V. & Wood, P. B.) 45
Conjugation, orientation of, in metabolites of 4-chlorocatechol and 4-chlororesorcinol (Dodgson, K. S. & Williams, R. T.) 381
Constitution and plant growth, effect of 2-phenoxyethylamine derivatives (Jones, R. L., Metcalfe, T. P. & Sexton, W. A.) 143
Convulsive treatment (electrical) of mental patients and effect on cholinesterase levels (Early, D. F., Hemphill, R. E., Reiss, M. & Brummel, E.) 552
Copper, effect on acid phosphatases (Abul-Fadl, M. A. M. & King, E. J.) 51
Copper, effect on succinic oxidase system (Slater, E. C.) 14
Copper in heart-muscle preparation (Slater, E. C.) 1
Coproporphyrin in molluscan shells (Nicholas, R. E. H. & Comfort, A.) 208
Coproporphyrin, occurrence in mycobacteria (Todd, C. M.) 386
Cord, umbilical, enzyme activities of blood in (Jones R. E. H. & McCance, R. A.) 464
Cortin excretion by mental patients (Reiss, M., Hemphill, R. E., Gordon, J. J. & Cook, E. R.) 574
Corynebacterium diphtheriae, growth factors for (Chattaway, F. W., Dolby, D. E., Hall, D. A. & Happold, F. C.) 592
Corynebacterium diphtheriae, prosthetic groups of cytochromes present in (Rawlinson, W. A. & Hale, J. H.) 247
Corynebacterium 'Q', substances able to replace oleic acid as growth factor of (Pollock, M. R., Howard, G. A. & Boughton, B. W.) 417
Coumarins and hatching of potato eelworm (Marrian, D. H., Russell, P. B. & Todd, A. R.) 533
Countercurrent distribution, use for separation of serine and threonine (Elliott, D. F.) 429
Cozymase, inactivation by central nervous system preparations (McIlwain, H. & Rodnight, R.) 337
Cozymase synthesis by *Lactobacillus arabinosus* (Hughes, D. E. & Williamson, D. H.) xxxvi
Cress seedlings, factors affecting synthesis of ascorbic acid in (Mapson, L. W., Cruickshank, E. M. & Chen, Yu-Tuan) 171
Crustacea, marine, astaxanthin distribution in (Goodwin, T. W. & Srisukh, S.) 268
Cucurbita pepo (squash), glutamic decarboxylase of (Hughes, D. E.) 325
*Cup plate assay of vitamin B_{12} (Cuthbertson, W. F. J. & Lloyd, J. T.) xvii
Cupric salts, effect on succinic dehydrogenase and succinic oxidase systems (Slater, E. C.) 130
Cyanide, effect on renal function (Nicholson, T. F.) 112
Cyanide, effect on succinic dehydrogenase and succinic oxidase systems (Slater, E. C.) 130
Cyanides, reaction of haems with (Keilin, J.) 440
*iso*Cyanides, reaction of haems with (Keilin, J.) 440
Cyanophenylglucuronides, *o*-, *m*- and *p*-, biosynthesis of (Smith, J. N.) 638
Cysteic acid decarboxylase activities of extract of rat liver (Sloane-Stanley, G. H.) 556
Cystophora cristata (hooded seals), vitamin A and liver fat of (Rodahl, K. & Davies, A. W.) 408
Cysts, eelworm hatching of (Calam, C. T., Raistrick, H. & Todd, A. R.) 513
Cytochrome, occurrence in mycobacteria (Todd, C. M.) 386
Cytochrome *b*, respiratory catalyst required for the reduction of cytochrome *c* by (Slater, E. C.) 14
Cytochrome *c*, respiratory catalyst required for reduction by cytochrome *b* (Slater, E. C.) 14
Cytochromes and succinic oxidase system (Slater, E. C.) 14
Cytochromes present in *Corynebacterium diphtheriae*, prosthetic groups of (Rawlinson, W. A. & Hale, J. H.) 247
Cytochrome-succinic dehydrogenase system, in heart muscle and in kidney (Slater, E. C.) 1
Cytoplasm, rabbit-liver, phospholipin metabolism in (Ada, G. L.) 422

Daucus carota (carrot), glutamic decarboxylase of (Hughes, D. E.) 325
DDT *see* 1:1:1-Trichloro-2:2-di(*p*-chlorophenyl)ethane
Deamination of glycine by α-radiation from disintegration of boron in a nuclear reactor (Dale, W. M., Davies, J. V. & Gilbert, C. W.) 543
Deamination of nitrogenous compounds by X-radiation (Dale, W. M., Davies, J. V. & Gilbert ,C. W.) 93
Deamination of threonine in the rat, irreversibility of (Elliott, D. F. & Neuberger, A.) xiii
Decarboxylase, bacterial glutamic, acceleration by cetyltrimethylammonium bromide (Hughes, D. E.) 325
Decarboxylases, amino-acid, of rat liver (Sloane-Stanley, G. H.) 556
Defectives, mental, cholinesterase of (Early, D. F., Hemphill, R. E., Reiss, M. & Brummel, E.) 552
Degeneration in central nervous system and vitamin A deficiencies in rats (Coetzee, W. H. K.) 628
Degeneration, Wallerian, lipid content of peripheral nerve during (Johnson, A. C., McNabb, A. R. & Rossiter, R. J.) 500
Dehydrogenase (succinic)-cytochrome system in heart muscle and in kidney (Slater, E. C.) 1
Dehydrogenase, succinic, effect of various inhibitors on (Slater, E. C.) 130
7-Dehydrosteroid in the frog (Morton, R. A. & Rosen, D. G.) 612
Dental enamel, human, nitrogen in (Anderson, D. J.) 31
Detoxication, characterization of phenylglucuronide (Garton, G. A., Robinson, D. & Williams, R. T.) 65
Detoxication; fates of phenol, phenylsulphuric acid and phenylglucuronide in relation to metabolism of benzene in rabbit (Garton, G. A. & Williams, R. T.) 158
Detoxication, studies in; biosynthesis of cyanophenylglucuronides (Smith, J. N.) 638
Di-2-chloroethyl sulphide and protein, antisera against complexes of (Fleming, D. S., Moore, A. M. & Butler, G. C.) 546
Dicyan-haem, resemblance to dihydroxylhaem (Keilin, J.) 448
Diet, effect on intestinal phosphatase of rat (Lawrie, N. R. & Yudkin, J.) 438

Diet, fatty acids of, effect on composition and turnover rates of liver phospholipins (Campbell, I. G., Olley, J. & Blewett, M.) 105
Diets, native, possible importance of locusts as source of vitamin A (Goodwin, T. W.) 472
Dietary protein, influence of levels on experimental liver injury in dogs (Bardawill, C. J. & Gornall, A. G.) iii
Diffusiometer, Gouy, polydisperse material in (Ogston, A. G.) 189
Diffusion constant, arithmetic mean, calculation for polydisperse materials (Ogston, A. G.) 189
Diffusion of polysaccharides from *Penicillium luteum* (Ogston, A. G.) 189
Digestibility of nitrogen of potato (Chick, A. & Slack, E. B.) 211
Dihydrocoenzymes and coenzyme-inactivating enzyme (McIlwain, H. & Rodnight, R.) 337
Dihydroxyl-haem, properties and nature (Keilin, J.) 448
3:4-Dihydroxyphenylalanine decarboxylase activities of extracts of rat liver and kidney (Sloane-Stanley, G. H.) 556
2:3-Dimercaptopropanol, effect on succinic dehydrogenase and succinic oxidase systems (Slater, E. C.) 130
2:3-Dimercaptopropanol, effect on succinic oxidase system (Slater, E. C.) 14
Dimethylacetothetin, mobility of methyl group in (Challenger, F. & Fothergill, P.) xxvii
N-2:4-Dinitrophenyl amino-acids, use of buffered columns in chromatographic separation of (Blackburn, S.) 579
N-2:4-Dinitrophenyl derivatives of methyl esters of amino-acids, separation by adsorption chromatography (Lowther, A. G. & Reith (Reich), W. S.) vi
Disaggregating enzyme of *Byssochlamys fulva* (Beaven, G. H. & Brown, F.) 221
Displacement chromatography on synthetic ion-exchange resins (Partridge, S. M.) 459
Diurnal variation of 17-ketosteroid excretion in mental patients (Reiss, M., Hemphill, R. E., Gordon, J. J. & Cook, E. R.) 574
Dog, experimental liver injury, influence of dietary protein levels on (Bardawill, C. J. & Gornall, A. G.) iii
Donnan equilibrium between maternal serum and amniotic fluid (McDougall, E. I.) 397
Dosimetry of reactions in nuclear reactors (Dale, W. M., Davies, J. V. & Gilbert, C. W.) 543

'Early lag', influence of amino-acids, and compounds in the Krebs oxidation cycle (Dagley, S., Dawes, E. A. & Morrison, G. A.) xxvi
'Early lag' phase of *Aerobacter aerogenes* (Dagley, S., Dawes, E. A. & Morrison, G. A.) xxv
Eclepic acid, eelworm hatching factor (Calam, C. T., Todd, A. R. & Waring, W. S.) 520
Eclepic acid, nature of (Marrian, D. H., Russell, P. B., Todd, A. R. & Waring, W. S.) 524
Eelworm, potato, hatching factor (Calam, C. T., Raistrick, H. & Todd, A. R.) 513; (Calam, C. T., Todd, A. R. & Waring, W. S.) 520; (Marrian, D. H., Russell, P. B., Todd, A. R. & Waring, W. S.) 524; (Russell, P. B., Todd, A. R. & Waring, W. S.) 528, 530; (Marrian, D. H., Russell, P. B. & Todd, A. R.) 533
Egg albumin, isolation of glucosamine and histidine by displacement chromatography from hydrolysate (Partridge, S. M.) 459
Eggs, hen, acid-soluble phosphorus in, during development of embryo (Ramsay, W. N. M.) ix
Eggs, hen, iron metabolism during development of embryo (Ramsay, W. N. M.) viii
Eggs, lobster, astaxanthin in (Goodwin, T. W. & Srisukh, S.) 268
Eggs, locust, β-carotene in (Goodwin, T. W. & Srisukh, S.) 263
Elaidic acid and growth of *Corynebacterium* 'Q' (Pollock, M. R., Howard, G. A. & Boughton, B. W.) 417
Elastin, amino-acid distribution of (Bowes, J. H. & Kenten, R. H.) 281
Elastin, preparation from ligamentum nuchae (Bowes, J. H. & Kenten, R. H.) 281
Electroconvulsive treatment of mental patients and effect on cholinesterase levels (Early, D. F., Hemphill, R. E., Reiss, M. & Brummel, E.) 552
Electrolysis, micro-, of water, apparatus for (Bentley, R.) 591
Electrolyte mixtures, fractionation by ion-exchange resins (Davies, C. W.) 38
Electrolytes, effect on molecular weight of tropomyosin (Adair, G. S., Bailey, K. & Tsao, T. C.) v
Electrophoresis, mobility of colloids in differential method for detection of small differences (Hoch, H.) 285
Embryo, acid-soluble phosphorus in hen eggs during development of (Ramsay, W. N. M.) ix
Embryo, iron metabolism of hen eggs during development of (Ramsay, W. N. M.) viii
Embryo muscle of calf intracellular fluid of (Gordon, A. H.) 99
Enamel, human dental, nitrogen in (Anderson, D. J.) 31
Energy of activation of the tryptophanase-tryptophan indole reaction (Happold, F. C. & Morrison, G. A.) xx
Enzyme activities in blood of infants and adults (Jones, P. E. H. & McCance, R. A.) 464
Enzyme, degradation of coenzymes I and II by (McIlwain, H. & Rodnight, R.) 337
Enzyme, disaggregating, in *Byssochlamys fulva* (Beaven, G. H. & Brown, F.) 221
Enzyme preparation from *Helianthus tuberosus* L. (Jerusalem artichoke), action on the carbohydrates present in the tubers (Edelman, J. & Bacon, J. S. D.) xxix
Enzyme system, cell-free, which accomplishes ring cleavage, preparation and properties of (Parr, W. H., Evans, R. A. & Evans, W. C.) xxix
Enzymes, pectic, of *Byssochlamys fulva* (Beaven, H. G. & Brown, F.) 221
Erucic acid and growth of *Corynebacterium* 'Q' (Pollock, M. R., Howard, G. A. & Boughton, B. W.) 417
Erythrocytes, acid phosphatases of (Abul-Fadl, M. A. M. & King, E. J.) 51
Escherichia coli, biosynthesis of nicotinamide by (Ellinger, P. & Abdel Kader, M. M.) 276
Escherichia coli, factors influencing polysaccharide content of (Dagley, S. & Dawes, E. A.) xxvi
Escherichia coli, glutamic decarboxylase and glutaminase from (Hughes, D. E.) 325
Escherichia coli, nucleic acid metabolism of (Stephenson, M. & Moyle, J. M.) vii
Escherichia coli, polysaccharide content of (Dagley, S. & Dawes, E. A.) 331
Ethanolamine, labelled, ^{15}N content of porphyrins following feeding (Muir, H. M. & Neuberger, A.) 163
Ewes fed during pregnancy, composition of allantoic and amniotic fluids during gestation (McDougall, E. I.) 397
Excretion of DL-thyroxine labelled with radioactive iodine (Clayton, J. C., Free, A. A., Page, J. E., Somers, G. F. & Woollett, E. A.) xx
Explants, chick-heart, grown *in vitro*, chemical composition of (Davidson, J. N. & Leslie, I.) xv
Eyes, frog, vitamin A in (Morton, R. A. & Rosen, D. G.) 612
Eyes, locust, carotenoids of (Goodwin, T. W. & Srisukh, S.) 263

Faeces of normal and cholesterol-fed animals, distribution of lipids in (Thomson, R. O.) xiv
Fat determination in degenerating cat nerves (Johnson, A. C., McNabb, A. R. & Rossiter, R. J.) 500

Fat, liver, of seals (Rodahl, K. & Davies, H. W.) 408
Fat, seasonal variation in frog (Morton, R. A. & Rosen, D. G.) 612
Fat synthesis in mammary gland, possible mechanism for (Folley, S. J. & French, T. H.) 270
Fatty acid determination in degenerating cat nerves (Johnson, A. C., McNabb, A. R. & Rossiter, R. J.) 500
Fatty acids, long-chain unsaturated, as essential bacterial growth factors (Pollock, M. R., Howard, G. A. & Boughton, B. W.) 417
Fatty tissues of locusts, β-carotene in (Goodwin, T. W. & Srisukh, S.) 263
Fermentation process in tea manufacture (Roberts, E. A. H.) 538
Fin-whale milk, vitamin A and kitol in (Barua, R. K. & Morton, R. A.) 308
Fish-liver oils, vitamin A potencies of (Coetzee, W. H. K.) 628
Fissurellidae, pigments of (Comfort, A.) 199
Flame photometer, photoelectric (Domingo, W. R. & Klyne, W.) 400
Flora, intestinal, of rats, and nicotinamide metabolism Ellinger, P. & Abdel Kader, M. M.) 276
Fluid, intracellular, of calf embryo muscle (Gordon, A. H.) 99
Foetal fluids of sheep, composition during gestation (McDougall, E. I.) 397
Formamide and formic acid, hydrolysis by rabbit-liver preparation (Bray, H. G., James, S. P., Thorpe, W. V., Wasdell, M. R. & Wood, P. B.) 467
Formamide and formic acid, metabolism in rabbits (Bray, H. G., James, S. P., Thorpe, W. V., Wasdell, M. R. & Wood, P. B.) 467
Fractionation of weak electrolyte mixture by ion-exchange resins (Davies, C. W.) 38
Frog (*Rana temporaria*), carotenoids, vitamin A and 7-dehydrosteroid in (Morton, R. A. & Rosen, D. G.) 612
Fructosans, renal clearance of (Beattie, J. & Corcoran, A. C.) xv
Fructosans, structures of (Bell, D. J. & Palmer, A.) xiv
Fruits of walnut, apparent vitamin C in (Wokes, F. & Melville, R.) 343
Fumarate, and oxidation of pyruvate (Coxon, R. V., Liébecq, C. & Peters, R. A.) 320
Fumarate, transformation to oxaloacetate by *Clostridium saccharobutyricum*, Strain GR4 (Cohen-Bazire, G. & Cohen, G. N.) 41
Furan derivatives as artificial hatching agents for potato eelworm (Russell, P. B., Todd, A. R. & Waring, W. S.) 530

Galactose, neutral polysaccharide containing, from *Penicillium luteum* (Freeman, G. G. & Macpherson, C. S.) 179
Gasometric methods, use for examining diffusion of hydrogen ions through mucosa (Terner, C.) 150
Gastric acidity, reduction by back-diffusion of hydrogen ions through the mucosa (Terner, C.) 150
Gastric mucosa, transport of radioactive sodium and potassium ions through (Crane, E. E. & Davies, R. E.) xxiii
Gastrophilus intestinalis larvae, metabolism of (Laser, H. & Rothschild, Lord) 598
Germinating oat and rape seed, activity of substituted β-phenoxyethylamines against (Jones, R. L., Metcalfe, T. P. & Sexton, W. A.) 143
Germination of spore-bearing aerobes (Hills, G. M.) 353, 363
Gestation, composition of foetal fluids of sheep during (McDougall, E. I.) 397
Gland, mammary bovine, utilization of acetic acid by (McClymont, G. L.) i
Globin, denatured, effect on succinic dehydrogenase and succinic oxidase systems (Slater, E. C.) 130
Globulins of potato juice (Chick, H. & Slack, E. B.) 211
δ-Gluconolactone as product of action of notatin on glucose (Bentley, R. & Neuberger, A.) 584
Glucosamine, *N*-acetyl, breakdown by strains of streptococci (Rogers, H. J.) 87
Glucosamine, isolation from a protein hydrolysate (Partridge, S. M.) 459
Glucose, acidic polysaccharide containing, from *Penicillium luteum* (Freeman, G. C. & Macpherson, C. S.) 179
Glucose, effect on the metabolism of kidney slices from adult and newborn rats (Robinson, J. R.) 68
Glucose, effect on metabolism of rat mammary gland (Folley, S. J. & French, T. H.) 270
Glucose, oxidation by notatin (Bentley, R. & Neuberger, A.) 584
Glucuronic acid, conjugation of toluic acids and amides with, in rabbit (Bray, H. G., Thorpe, W. V. & Wood, P. B.) 45
Glucuronic acid, conjugation of xylene metabolites with (Bray, H. G., Humphris, B. G. & Thorpe, W. V.) 241
β-Glucuronidase, relationship to the glucuronide-synthesizing system in the mouse (Karunairatnam, M. C., Kerr, L. M. H. & Levvy, G. A.) 496
Glucuronide-synthesizing system in the mouse, relationship to β-glucuronidase (Karunairatnam, M. C., Kerr, L. M. H. & Levvy, G. A.) 496
Glucuronides from adrenaline and protocatechuic aldehyde in rabbit (Dodgson, K. S. & Williams, R. T.) 381
Glucuronides from rabbit urine after feeding 4-chlorocatechol and 4-chlororesorcinol (Dodgson, K. S. & Williams, R. T.) 381
Glucuronides of cyanophenols, biosynthesis in rabbit (Smith, J. N.) 638
Glutamic acid peptides as growth factors for *Corynebacterium diphtheriae* (Chattaway, F. W., Dolby, D. E., Hall, D. A. & Happold, F. C.) 592
Glutamic decarboxylase, bacterial, acceleration by cetyltrimethylammonium bromide (Hughes, D. E.) 325
Glutaminase, bacterial, acceleration by cetyltrimethylammonium bromide (Hughes, D. E.) 325
Glutathione, effect on succinic dehydrogenase and succinic oxidase systems (Slater, E. C.) 130
Glycerophosphate in wheat starch (Rooke, H. S., Lampitt, L. H. & Jackson, E. M.) 231
Glycerophosphates, α- and β-, hydrolysis by acid phosphatases (Abul-Fadl, M. A. M. & King, E. J.) 51
Glycine as terminal amino-acid of insulin (Sanger, F.) 563
Glycine, conjugation of toluic acids and amides with, in rabbit (Bray, H. G., Thorpe, W. V. & Wood, P. B.) 45
Glycine, conjugation of xylene metabolites with (Bray, H. G., Humphris, B. G. & Thorpe, W. V.) 241
Glycine, conversion of acetate to in rat (Arnstein, H. R. V. & Neuberger, A.) iii
Glycine, deamination by α-rays (Dale, W. M., Davies, J. V. & Gilbert, C. W.) 93, 543
Glycine, labelled, ^{15}N content of porphyrins following feeding (Muir, H. M. & Neuberger, A.) 163
Glycine, reaction with mustard gas (Fleming, D. S., Moore, A. M. & Butler, G. C.) 546
Glycogen, distribution in rabbit liver (Nys, A., Aubert, X. Duve, C. de) 245
Glyoxalase activity in erythrocytes of infants and adults (Jones, P. E. H. & McCance, R. A.) 464
Gouy diffusiometer, polydisperse material in (Ogston, A. G.) 189
Granules from rabbit-liver cytoplasm, analysis of (Ada, G. L.) 422
Growth factors, bacterial, long-chain unsaturated fatty acids as (Pollock, M. R., Howard, G. A. & Boughton, B. W.) 417

Growth factors essential for *Lactobacillus leichmanii* 313 and *Leuconostoc citrovorum*, microbiological assay of (Lees, K. A. & Emery, W. B.) ii
Growth factors for *Corynebacterium diphtheriae* (Chattaway, F. W., Dolby, D. E., Hall, D. A. & Happold, F. C.) 592
Growth of plants, relationship between constitution and effect of chemical compounds (Jones, R. L., Metcalfe, T. P. & Sexton, W. A.) 143
Growth test, comparison with histological method for determination of vitamin A (Coetzee, W. H. K.) 628
Guinea pig-liver extract, 3:4-dihydroxyphenylalanine oxidase activity of (Sloane-Stanley, G. H.) 556

Haematin compounds, and succinic oxidase system (Slater, E. C.) 14
Haematin compounds in succinic oxidase preparations (Slater, E. C.) 1
Haematinic acid, dissociation constant, synthesis and ultra-violet absorption of (Muir, H. M. & Neuberger, A.) 163
Haematohaem, dihydroxyl compounds of (Keilin, J.) 448
Haemin of rabbits fed glycine and ethanolamine containing ^{15}N (Muir, H. M. & Neuberger, A.) 163
Haemochromogen, alkaline (Keilin, J.) 448
Haemolytic substance, effect on succinic oxidase activity (Slater, E. C.) 8
Haems, dihydroxyl, magnetic susceptibility of (Keilin, J.) 448
Haems of *Corynebacterium diphtheriae* (Rawlinson, W. A. & Hale, J. H.) 247
Haems, reaction with cyanides and *iso*cyanides (Keilin, J.) 440
Halichoerus grypus (Atlantic seal), vitamin A and liver fat of (Rodahl, K. & Davies, A. W.) 408
Haliotis cracherodii, indigoid pigments of (Comfort, A.) 204
Hatching agent, artificial, for potato eelworm (Calam, C. T., Todd, A. R. & Waring, W. S.) 520
Hatching factor, potato eelworm (Calam, C. T., Raistrick, H. & Todd, A. R.) 513; (Calam, C. T., Todd, A. R. & Waring, W. S.) 520; (Marrian, D. H., Russell, P. B., Todd, A. R. & Waring, W. S.) 524; (Russell, P. B., Todd, A. R. & Waring, W. S.) 528; 530; (Marrian, D. H., Russell, P. B. & Todd, A. R.) 533
Heart explants, chick, grown *in vitro*, chemical composition of (Davidson, J. N. & Leslie, I.) xv
Heart-muscle preparations, succinic oxidase system (Slater, E. C.) 8, 14
Heart-muscle, succinic dehydrogenase-cytochrome system in (Slater, E. C.) 1
Heart, rat, enzyme activities and effect of vitamins (Bhagvat, K. & Devi, P.) 32
Helianthus tuberosus L. (Jerusalem artichoke), enzyme preparation from action on carbohydrates present in the tubers (Edelman, J. & Bacon, J. S. D.) xxix
Hen eggs, acid-soluble phosphorus in, during development of embryo (Ramsay, W. N. M.) ix
Hen eggs, iron metabolism during development of embryo (Ramsay, W. N. M.) viii
Hepatopancreas of lobster, β-carotene in (Goodwin, T. W. & Srisukh, S.) 268
Herbicidal activity of phenoxyethylamines (Jones, R. L., Metcalfe, T. P. & Sexton, W. A.) 143
Heterodera rostochiensis Wollenweber *see* Potato eelworm
Hexamethylenetetramine, herbicidal activity of derivatives (Jones, R. L., Metcalfe, T. P. & Sexton, W. A.) 143
Hexose content of cress seedlings, and ascorbic acid content Mapson, L. W., Cruickshank, E. M. & Chen, Yu-Tuan) 171
Hibernation, carotenoids in (Morton, R. A. & Rosen, D. G.) 612
Histidine as an essential metabolite of *Bacterium aerogenes* (Devi, P. & Higginbottom, C.) 391
Histidine, isolation from a protein hydrolysate (Partridge, S. M.) 459
Histological method for the biological estimation of vitamin A (Coetzee, W. H. K.) 628
Homarus vulgaris Edw., astaxanthin in (Goodwin, T. W. & Srisukh, S.) 268
Hormones, anterior pituitary, and mammary gland metabolism (Folley, S. J. & French, T. H.) 270
Horse-radish root extracts, oxidation of manganese by (Kenten, R. H. & Mann, P. J. G.) 255
Hydrogen ions, back diffusion through the mucosa, reduction of gastric acidity by (Terner, C.) 150
Hydrogen peroxide, and oxidation of manganese by plant extract (Kenten, R. H. & Mann, P. J. G.) 255
Hydrogen peroxide, effect on succinic oxidase system (Slater, E. C.) 14
Hydrogen peroxide, oxidation of manganese by plant extracts in the presence of (Kenten, R. H. & Mann, P. J. G.) 255
Hydrolysis of phenylglucuronide (Garton, G. A., Robinson, D. & Williams, R. T.) 65
Hydrolysis of proteins (Flowers, H. M. & Reith (Reich), W. S.) xx
Hydroxy-amines, respiration of rat-liver slices in presence of (Long, C.) xi
Hydroxyamino-acids, separation from proteins and hydrolysates (Elliott, D. F.) 429
2-Hydroxy-2′-glycinodiethyl sulphide from mustard gas and ethyl aminoacetate (Fleming, D. S., Moore, A. M. & Butler, G. C.) 546
Hydroxylamine, reduction by *Clostridium saccharobutyricum*, strain GR4 (Cohen-Bazire, G. & Cohen, G. N.) 41
Hydroxylysine, in calf embryo muscle (Gordon, A. H.) 99
Hypodermis of lobster and prawn, astaxanthin in (Goodwin, T. W. & Srisukh, S.) 268
Hypoproteinaemia, plasma and tissue protein regeneration in renal disease with (Herbert, F. K.) xxii

Imino ethers from amino-acid esters (Elliott, D. F.) 429
Inactivation of serum alkaline phosphatase by adrenaline and related substances (Anderson, A. B.) xxxvi
Inanition, carotenoids in (Morton, R. A. & Rosen, D. G.) 612
Indicator yellow and retinene, (Ball, S., Collins, F. D., Dalvi, P. D. & Morton, R. A.) 304
Indigoid pigments of *Haliotis cracherodii* (Comfort, A.) 204
Indole reaction, tryptophanase-tryptophan, energy of activation of (Happold, F. C. & Morrison, G. A.) xx
Indophenol, reaction with apparent vitamin C (Wokes, F. & Melville, R.) 343
Infants, enzyme activities in blood of (Jones, P. E. H. & McCance, R. A.) 464
Inhibition of plant phosphorylases by β-amylase (Porter, H. K.) xxxvii
Inhibitors, action on the system of enzymes which catalyse the aerobic oxidation of succinate (Slater, E. C.) 8
Inhibitors of succinic dehydrogenase and succinic oxidase systems (Slater, E. C.) 130
Inorganic phosphate, formation from adenosinetriphosphate on storage (Bailey, K.) 479
Inorganic pyrophosphate, formation from adenosinetriphosphate on storage (Bailey, K.) 479
Insulin, terminal peptides of (Sanger, F.) 563
Integument of locusts, carotenoids of (Goodwin, T. W. & Srisukh, S.) 263
Interference of cations in flame photometer (Domingo, W. R. & Klyne, W.) 400
Intestinal flora of rats and nicotinamide metabolism (Ellinger, P. & Abdel Kader, M. M.) 276
Intestinal phosphatase of rat, effect of diet on (Lawrie, N. S. & Yudkin, J.) 438
Intracellular fluid of calf embryo muscle (Gordon, A. H.) 99

Intracellular mode of action of sulphonamide derivatives (Bell, E. A., Cocker, W. & O'Meara, R. A. Q.) 373
Inulins, structure of (Bell, D. J. & Palmer, A.) xiv
Involution in the rat, respiration and acid production of the mammary tissue during (Folley, S. J. & French, T. H.) 270
Iodinated casein, alleged formation of vitamin A from β-carotene treated with (Cama, H. R. & Goodwin, T. W.) 317
Iodine, radioactive, absorption and excretion of DL-thyroxine labelled with (Clayton, J. C., Free, A. A., Page, J. E., Somers, G. F. & Wollett, E. A.) xx
o-Iodosobenzoate, effect on succinic dehydrogenase and succinic oxidase systems (Slater, E. C.) 130
Ion-exchange resins, displacement chromatography on (Partridge, S. M.) 459
Ion-exchange resins, fractionation of weak electrolyte mixtures (Davies, C. W.) 38
Ionic yields in radiation processes, calculation of (Dale, W. M., Davies, J. V. & Gilbert, C. W.) 93
Irisin, structure of (Bell, D. J. & Palmer, A.) xiv
Iron, effect on acid phosphatases (Abul-Fadl, M. A. M. & King, E. J.) 51
Iron metabolism of hen eggs during development of embryo (Ramsay, W. N. M.) viii

Juglans regia, (walnut), apparent vitamin C in (Wokes, F. & Melville, R.) 343
Juglone-like compound from apparent vitamin C (Wokes, F. & Melville, R.) 343

Kephalin determination in degenerating cat nerves (Johnson, A. C., McNabb, A. R. & Rossiter, R. J.) 500
α-Ketoglutarate, formation during oxidation of pyruvate by pigeon brain (Coxon, R. V., Liébecq, C. & Peters, R. A.) 320
Kidney, dog, perfusion *in situ* (Nicholson, T. F.) 112
Kidney lesions, unilateral, effect on renal function (Nicholson, T. F.) 112
Kidney, mouse, glucuronide-synthesizing system in (Karunairatnam, M. C., Kerr, L. M. H. & Levvy, G. A.) 496
Kidney of frog, vitamin A in (Morton, R. A. & Rosen, D. G.) 612
Kidney preparations, succinic oxidase system (Slater, E. C.) 14
Kidney, rat, 3:4-dihydroxyphenylalanine decarboxylase activity of (Sloane-Stanley, G. H.) 556
Kidney, rat, enzyme activities and effect of vitamins in (Bhagvat, K. & Devi, P.) 32
Kidney slices from adult and newborn rats, effects of glucose and calcium on (Robinson, J. R.) 68
Kidney, succinic dehydrogenase-cytochrome system in (Slater, E. C.) 1
Kitol esters, preparation from whale liver oil (Barua, R. K. & Morton, R. A.) 308
Krebs oxidation cycle, compounds in, influence on 'early lag' (Dagley, S., Dawes, E. A. & Morrison, G. A.) xxvi
Krill, preformed vitamin A in (Kon, S. K. & Thompson, S. Y.) xxxi

Lactate, formation from *N*-acetylglucosamine by streptococci (Rogers, H. J.) 87
Lactation in the rat, respiration and acid production of mammary tissue during (Folley, S. J. & French, T. H.) 270
Lactones of furan derivatives and potato eelworm hatching factor (Russell, P. B., Todd, A. R. & Waring, W. S.) 530
Lactose, estimation in presence of other sugars (Malpress, F. H. & Morrison, A. B.) 455
Lactobacillus arabinosus, cozymase synthesis by (Hughes, D. E. & Williamson, D. H.) xxxvi
Lactobacillus casei, improved medium for microbiological assays with (Kodicek, E. & Mistry, S. P.) xxxv
Lactobacillus leichmanii, microbiological assay of growth factors essential for (Lees, K. A. & Emery, W. B.) ii
Laevulinic acid derivatives and hatching factor of potato eelworm (Russell, P. B., Todd, A. R. & Waring, W. S.) 530
Lag phase of *Aerobacter aerogenes* (Dagley, S., Dawes, E. A. & Morrison, G. A.) xv
Larvae, *Gastrophilus intestinalis*, metabolism of (Laser, H. & Rothschild, Lord) 598
Lauric acid and growth of *Corynebacterium* 'Q' (Pollock, M. R., Howard, G. A. & Boughton, B. W.) 417
Leaves of walnut, apparent vitamin C in (Wokes, F. & Melville, R.) 343
Lecithin, determination in degenerating cat nerves (Johnson, A. C., McNabb, A. R. & Rossiter, R. J.) 500
Leuconostoc citrovorum, microbiological assay of growth factors essential for (Lees, K. A. & Emery, W. B.) ii
Levan, grass, structure of (Bell, D. J. & Palmer, A.) xiv
Ligamentum nuchae, preparation of elastin from (Bowes, J. H. & Kenten, R. H.) 281
Linoleic acid and growth of *Corynebacterium* 'Q' (Pollock, M. R., Howard, G. A. & Boughton, B. W.) 417
Linolenic acid and growth of *Corynebacterium* 'Q' (Pollock, M. R., Howard, G. A. & Boughton, B. W.) 417
Lipid content of peripheral nerve during Wallerian degeneration (Johnson, A. C., McNabb, A. R. & Rossiter, R. J.) 500
Lipid determination in degenerating cat nerves (Johnson, A. C., McNabb, A. R. & Rossiter, R. J.) 500
Lipid in rabbit liver (Ada, G. L.) 422
Lipids, distribution in faeces of normal and cholesterol-fed animals (Thomson, R. O.) xiv
Lipids of rats, on diets containing pure fatty acids (Campbell, I. G., Olley, J. & Blewett, M.) 105
Lipids of whale liver (Barua, R. K. & Morton, R. A.) 308
Liver fat of seals (Rodahl, K. & Davies, A. W.) 408
Liver injury in dogs, experimental, influence of dietary protein levels on (Bardawill, C. J. & Gornall, A. G.) iii
Liver, mouse, glucuronide-synthesizing system in (Karunairatnam, M. C., Kerr, L. M. H. & Levvy, G. A.) 496
Liver of frog, vitamin A in (Morton, R. A. & Rosen, D. G.) 612
Liver oils, fish, vitamin A potencies of (Coetzee, W. H. K.) 628
Liver oil, whale, analysis of (Barua, R. K. & Morton, R. A.) 308
Liver phospholipins, effect of dietary oleic and palmitic acid on the composition and turnover rates of (Campbell, I. G., Olley, J. & Blewett, M.) 105
Liver, rabbit, distribution of glycogen in (Nys, A., Aubert, X. & Duve, C. de) 245
Liver, rat, amino-acid decarboxylases of (Sloane-Stanley, G. H.) 556
Liver, rat, enzyme activities and effect of vitamins (Bhagvat, K. & Devi, P.) 32
Liver, rat, vitamin concentrations in (Bhagvat, K. & Devi, P.) 32
Liver slices, rat, respiration in presence of aliphatic amines, hydroxy-amines and quaternary ammonium salts (Long C.) xi
Lobster, astaxanthin in (Goodwin, T. W. & Srisukh, S.) 268
Locusta migratoria migratorioides R. & F., carotenoid distribution in (Goodwin, T. W.) 472
Locusta migratoria migratorioides R. & F., carotenoids of the integument of (Goodwin, T. W. & Srisukh, S.) 263
Locusts, biochemistry of (Goodwin, T.) 472
Locusts, carotenoids of integument of (Goodwin, T. W. & Srisukh, S.) 263

Logarithmic dose response curves, potency calculation for use with (Basil, B.) xvii
Lymph nodes, preparation of reticular tissue from (Bowes, J. H. & Kenten, R. H.) 281

Maclurin, effect on capillary resistance in guinea pig (Zilva, S. S.) 79
Magnesium, effect on acid phosphateses (Abul-Fadl, M. A. M. & King, E. J.) 51
Magnesium in foetal fluids of sheep during gestation (McDougall, E. I.) 397
Magnetic susceptibility of dihydroxyl haems (Keilin, J.) 448
Malate, fermentation by *Clostridium saccharobutyricum*, strain GR4 (Cohen-Bazire, G. & Cohen, G. N.) 41
Mammals, metabolism of alveolar bone in (Weidmann, S. & Rogers, H. J.) xxv
Mammary gland, bovine, utilization of acetic acid by (McClymont, G. L.) i
Mammary gland, intermediary metabolism (Folley, S. J. & French, T. H.) 117, 270
Manganese, oxidation by plant extracts in the presence of hydrogen peroxide (Kenten, R. H. & Mann, P. J. G.) 255
Manic-depressive patients, ketosteroid excretion of (Reiss, M., Hemphill, R. E., Gordon, J. J. & Cook, E. R.) 574
Manometric method for determination of respiratory quotients (Laser, H. & Rothschild, Lord) 598
Marine crustacea, astaxanthin distribution in (Goodwin, T. W. & Srisukh, S.) 268
Matrix, organic, of bone (Rogers, H. J.) xxiv
Mechanism of bacterial oxidation of aromatic compounds (Parr, W. H., Evans, R. A. & Evans, W. C.) xxix
Medium, phosphate buffered, for uses with kidney slices (Robinson, J. R.) 68
Mental defectives, cholinesterase of (Early, D. F., Hemphill, R. E., Reiss, M. & Brummel, E.) 552
Mental patients, urinary steroid excretion of (Reiss, M. Hemphill, R. E., Gordon, J. J. & Cook, E. R.) 574
Mercurialis perennis L., micro-estimation and origin of methylamine in (Cromwell, B. T.) 84
Mesoporphyrin of rabbits fed glycine and ethanolamine containing ^{15}N (Muir, H. M. & Neuberger, A.) 163
Metabolism, intermediary, of mammary gland (Folley, S. J. & French, T. H.) 117, 270
Metabolism of the alveolar bone of mammals (Weidmann, S. & Rogers, H. J.) xxv
Methyl esters of amino-acids, *N*-2:4-dinitrophenyl derivatives of, separation by adsorption chromatography (Lowther, A. G. & Reith (Reich), W. S.) vi
Methyl group in dimethylacetothetin, mobility of (Challenger, F. & Fothergill, P.) xxvii
Methylamine, micro-estimation and origin in *Mercurialis perennis* L. (Cromwell, B. T.) 84
Methylene blue, reduction by succinate (Slater, E. C.) 1
Methylethanolamine as a probable precursor of methylamine (Cromwell, B. T.) 84
Methylethylmaleic acid, dissociation constant, synthesis, ultraviolet absorption of (Muir, H. M. & Neuberger, A.) 163
N-Methyl-2-pyridone-3-(and -5-)carboxylamide in human urine, determination of (Holman, W. I. M. & Lange, D. J. de) 559
Microbiological assay of growth factors essential for *Lactobacillus leichmanii* and *Leuconostoc citrovorum* (Lees, K. A. & Emery, W. B.) ii
Microbiological assay of substances able to replace oleic acid as growth factor of *Corynebacterium* 'Q' (Pollock, M. R., Howard, G. A. & Boughton, B. W.) 417
Microbiological assays with *Lactobacillus casei*, improved medium for (Kodicek, E. & Mistry, S. P.) xxxv
Micro-electrolysis of water, apparatus for (Bentley, R.) 591
Microsomes from rabbit-liver cytoplasm, analysis of (Ada, G. L.) 422
Milk, effect of thyroxine and thiouracil on water-soluble vitamins in (Chanda, R., McNaught, M. L. & Owen, E. C.) xix
Milk of fin whale (Barua, R. K. & Morton, R. A.) 308
Mobilization, alleged, of vitamin A by adrenaline (Goodwin, T. W. & Wilson, A. A.) 370
Mobility of colloids in electrophoresis, differential method for detection of small differences (Hoch, H.) 285
Mobility of methyl group in dimethylacetothetin, (Challenger, F. & Fothergill, P.) xxvii
Modulators, photopic, and products from vitamin A and retinene (Ball, S. & Morton, R. A.) 298
Molecular weight of tropomyosin, effect of electrolytes on (Adair, G. S., Bailey, K. & Tsao, T. C.) v
Molluscan shells, acid-soluble pigments of (Comfort, A.) 199, 204, 208
Molybdate complex of *dextro-iso*citric acid, optical rotation of (Eggleston, L. V. & Krebs, H. A.) 578
Mono-amine oxidase activity of placenta and uterus (Thompson, R. H. S. & Tickner, A.) 125
Monoaminophospholipin determination in degenerating cat nerves (Johnson, A. C., McNabb, A. R. & Rossiter, R. J.) 204, 500
Mould, *Byssochlamys fulva*, pectic enzymes of (Beaven, G. H. & Brown, F.) 221
Mouse, glucuronide-synthesizing system in (Karunairatnam, M. C., Kerr, L. M. H. & Levvy, G. A.) 496
Mucosa, back-diffusion of hydrogen ions through (Terner, C.) 150
Mucosa, gastric, transport of radioactive sodium and potassium ions through (Crane, E. E. & Davies, R.E.) xxiii
Muscle, calf embryo, intracellular fluid (Gordon, A. H.) 99
Muscle, content of adenosinediphosphate (Bailey, K.) 479
Muscle, heart, activity of succinic oxidase system (Slater, E. C.) 8
Muscle, heart, succinic dehydrogenase-cytochrome system in (Slater, C. E.) 1
Mustard gas *see* Di-2-chloroethyl sulphide
Mycobacteria, occurrence of cytochrome and coproporphyrin (Todd, C. M.) 386
Myelin, lipid determination in degenerating cat nerves (Johnson, A. C., McNabb, A. R. & Rossiter, R. J.) 500
Myelin substance, degeneration in vitamin A deficiency (Coetzee, W. H. K.) 628
Myokinase in assay of adenosinediphosphate and adenosinetriphosphate (Bailey, K.) 479
Myosin adenosinetriphosphatase in assay of adenosinetriphosphate and adenosinediphosphate (Bailey, K.) 479
Myristic acid and growth of *Corynebacterium* 'Q' (Pollock, M. R., Howard, G. A. & Boughton, B. W.) 417

Nephrops norvegicus L., astaxanthin in (Goodwin, T. W. & Srisukh, S.) 268
Nerve, peripheral, lipid content during Wallerian degeneration (Johnson, A. C., McNabb, A. R. & Rossiter, R. J.) 500
Nerve, sciatic, of cat, lipids of (Johnson, A. C., McNabb, A. R. & Rossiter, R. J.) 500
Nervous system, central, degeneration in, and vitamin A deficiencies in rats (Coetzee, W. H. K.) 628
Nervous system, central, degradation of coenzymes I and II by an enzyme from (McIlwain, H. & Rodnight, R.) 337
Neurospora crassa, amino-acid oxidase (Bender, A. E., Krebs, H. A. & Horowitz, N. H.) xxi
Newborn animals, cholinesterase activity of serum of (McCance, R. A., Hutchinson, A. O., Dean, R. F. A. & Jones, P. E. H.) 493

Nicotinamide glycosides and coenzyme-inactivating enzyme (McIlwain, H. & Rodnight, R.) 337
Nicotinamide liberation during inactivation of coenzyme II (McIlwain, H. & Rodnight, R.) 337
Nicotinamide metabolism, effect of tryptophan (Ellinger, P. & Abdel Kader, M. M.) 276
Nicotinic acid as an essential metabolite of *Bacterium aerogenes* (Devi, P. & Higginbottom, C.) 391
Nicotinic acid metabolism (Holman, W. I. M. & Lange, D. J. de) 559
Nicotinic acid, modified chemical estimation of (Hughes, D. E.) xxxiv
Ninhydrin and destruction of amino-acids on chromatograms (Woiwood, A. J.) 412
Nisin, preparation of (Berridge, N. J.) 486
Nitrogen, amino, estimation of micro amounts and its application to paper partition chromatography (Woiwod, A. J.) 412
Nitrogen, distribution of ^{15}N in the ring system of porphyrins (Muir, H. M. & Neuberger, A.) 163
Nitrogen in human dental enamel (Anderson, D. J.) 31
Nitrogen, loss of, by rat kidney slices (Robinson, J. R.) 68
Nitrogen of substances from connective tissue (Bowes, J. H. & Kenten, R. H.) 281
Nitrogenous bases of potato juice (Chick, H. & Slack, E. B.) 211
Nitrogenous compounds, deamination of, by X-radiation (Dale, W. M., Davies, J. K. & Gilbert, C. W.) 93
Nitrogenous substances in potato, distribution and nutritive value of (Chick, H. & Slack, E. B.) 211
Nomadacris septemfasciata, astaxanthin in (Goodwin, T. W. & Srisukh, S.) 263
Non-protein nitrogen in foetal fluids of sheep during gestation (McDougall, E. I.) 397
Non-protein nitrogen of potato (Chick, H. & Slack, E.B.) 211
Notatin, mechanism of action of (Bentley, R. & Neuberger, A.) 584
Nuclear reactor, deamination of glycine in (Dale, W. M., Davies, J. V. & Gilbert, C. W.) 543
Nuclear reactor, α-radiation from the disintegration of boron in (Dale, W. M., Davies, J. V. & Gilbert, C. W.) 543
Nuclei, rabbit-liver, preparation of (Ada, G. L.) 422
Nucleic acid in rabbit liver (Ada, G. L.) 422
Nucleic acid metabolism of *Escherichia coli* (Stephenson, M. & Moyle, J. M.) vii
Nucleic acids, chromatography of (Markham, R. & Smith, J. D.) 294
Nucleosides in nucleic acids, identification and estimation of (Markham, R. & Smith, J. D.) 294
Nucleus, cell, phosphorus compounds in (Davidson, J. N. & McIndoe, W. M.) xvi
Nutritive value of the nitrogenous substances in the potato (Chick, H. & Slack, E. B.) 211

Oat seed, germinating, activity of substituted β-phenoxyethylamines against (Jones, R. L., Metcalfe, T. P. & Sexton, W. A.) 143
Octadec-2-enoic acid and growth of *Corynebacterium* 'Q' (Pollock, M. R., Howard, G. A. & Boughton, B. W.) 417
Oedema, plasma and tissue protein regeneration in renal disease with (Herbert, F. K.) xxii
Oestrogen excretion by mental patients (Reiss, M., Hemphill, R. E., Gordon, J. J. & Cook, E. R.) 574
Oil, whale liver, analysis of (Barua, R. K. & Morton, R. A.) 308
Oils, fish-liver, vitamin A potencies of (Coetzee, W. H. K.) 628
αβ-Oleic acid *see* Octadec-2-enoic acid
Oleic acid as an essential growth factor for *Corynebacterium* 'Q' (Pollock, M. R., Howard, G. A. & Boughton, B.W.) 417
Oleic acid, dietary, effect on composition and turnover rates of liver phospholipins (Campbell, I. G., Olley, J. & Blewett, M.) 105
Opisthobranch, tectibranch, pigments of (Comfort, A.) 199
Optical form of amino-acids, relation to biological activity in germination of *Bacillus anthracis* (Hills, G. M.) 363
Optical rotation of the molybdate complex of *dextro-isocitric* acid (Eggleston, L. V. & Krebs, H. A.) 578
Organic matrix of bone (Rogers, H. J.) xxiv
Organo-silicon compounds, naturally occurring (Holt, P. F. & Yates, D. M.) xxxii
Organs, frog, weight expressed as percentage of body weight (Morton, R. A. & Rosen, D. G.) 612
Ovaries in frog, carotenoids in (Morton, R. A. & Rosen, D. G.) 612
Ovaries in frog, dehydrosteroids in (Morton, R. A. & Rosen, D. G.) 612
Oxaloacetate, formation from fumarate by *Clostridium saccharobutyricum*, Strain GR4 (Cohen-Bazire, G. & Cohen, G. N.) 41
Oxazolines, use in isolation of threonine and serine from proteins (Elliott, D. F.) 429
Oxazolones and hatching of potato eelworm (Marrian, D. H., Russell, P. B. & Todd, A. R.) 533
Ox hide, preparation of collagen from (Bowes, J. H. & Kenten, R. H.) 281
Oxidase activity, mono-amine, of placenta and uterus (Thompson, R. H. S. & Tickner, A.) 125
Oxidase, amino-acid, of *Neurospora crassa* (Bender, A. E., Krebs, H. A. & Horowitz, N. H.) xxi
Oxidase, pyruvate, system in brain (Coxon, R. V., Liébecq, C. & Peters, R. A.) 320
Oxidase system, succinic, effect of sulphydryl-combining compounds on activity of (Slater, E. C.) 130
Oxidase system, succinic, of heart-muscle and kidney preparations (Slater, E. C.) 14
Oxidation of succinate, aerobic, action of inhibitors (Slater, E. C.) 8
Oxygen and oxidation of glucose by notatin (Bentley, R. & Neuberger, A.) 584
Oxygen uptake, manometric method for determination (Laser, H. & Rothschild, Lord) 598
Oxyhaemoglobin mobility in electrophoresis (Hoch, H.) 285

Palmitic acid and growth of *Corynebacterium* 'Q' (Pollock, M. R., Howard, G. A. & Boughton, B. W.) 417
Palmitic acid, dietary, effect on composition and turnover rates of liver phospholipins (Campbell, I. G., Olley, J. & Blewett, M.) 105
Palmitoleic acid and growth of *Corynebacterium* 'Q' (Pollock, M. R., Howard, G. A. & Boughton, B. W.) 417
Paper chromatography of the vitamin B_{12} group of factors (Lester Smith, E. & Cuthbertson, W. F. J.) xii
Paper partition chromatography, application of estimation of micro amounts of amino nitrogen (Woiwod, A. J.) 412
Paper partition chromatography of sugars in urine (Eastham, M.) xiii
Partition chromatography, paper, application of estimation of micro amounts of amino nitrogen to (Woiwod, A. J.) 412
Partition chromatography, paper, of sugars in urine (Eastham, M.) xii
Partition chromatography, separation of penicillins by (Dobson, F. & Stroud, S. W.) i
Partition chromatography, use in separation of serine and threonine (Elliott, D. F.) 429
Pea (*Pisum sativum*), chemical changes in carbohydrates during growth of (Eastham, M.) xvii

Pectic enzymes of *Byssochlamys fulva* (Beaven, G. H. & Brown, F.) 221
Penicillins, separation by partition chromatography (Dobson, F. & Stroud, S. W.) i
Penicillium luteum, sedimentation and diffusion of polysaccharides from (Ogston, A. G.) 189
Penicillium luteum series, metabolic products of (Freeman, G. G. & Macpherson, C. S.) 179
Pentabromoacetone, in estimation of citric acid (Weil-Malherbe, H. & Bone, A. D.) 377
Peptide amino nitrogen, determination of micro amounts of (Woiwod, A. J.) 412
Peptides of glutamic acid, as growth factors for *Corynebacterium diphtheriae* (Chattaway, F. W., Dolby, D. E., Hall, D. A. & Happold, F. C.) 592
Peptides, terminal, of insulin (Sanger, F.) 563
Perfusion of dog kidney *in situ* (Nicholson, T. F.) 112
Peripheral blood, bovine, acetic acid in (McClymont, G. L.) i
Peripheral nerve during Wallerian degeneration, lipid content (Johnson, A. C., McNabb, A. R. & Rossiter, R. J.) 500
Peroxidase, and oxidation of manganese (Kenten, R. H. & Mann, P. J. G.) 255
Petroselinic acid and growth of *Corynebacterium* 'Q' (Pollock, M. R., Howard, G. A. & Boughton, B. W.) 417
Phenol, fate in rabbit (Garton, G. A. & Williams R. T.) 158
Phenoxyacetic acids, herbicidal effects, relationship to phenoxyethylamines (Jones, R. L., Metcalfe, T. P. & Sexton, W. A.) 143
Phenoxyacetic acids, possible formation from phenoxyethylamines by living plants (Jones, R. L., Metcalfe, T. P. & Sexton, W. A.) 143
2-Phenoxyethylamine derivatives, effect on plant growth (Jones, R. L., Metcalfe, T. P. & Sexton, W. A.) 143
Phenylacetamide, hydrolysis by rabbit-liver preparation (Bray, H. G., James, S. P., Thorpe, W. V., Wasdell, M. R. & Wood, P. B.) 467
Phenylalanine as terminal amino-acid of insulin (Sanger, F.) 563
Phenylalanine, effect on polysaccharide content of *Escherichia coli* (Dagley, S. & Dawes, E. A.) 331
Phenylglucuronide, characterization and rate of hydrolysis compared with that of phenylsulphuric acid (Garton, G. A., Robinson, D. & Williams, R. T.) 65
Phenylglucuronide, fate in rabbit (Garton, G. A. & Williams, R. T.) 158
Phenylglucuronidic acid, benzylamine salt of (Garton, G. A., Robinson, D. & Williams, R. T.) 65
Phenyl phosphate, hydrolysis by acid phosphatases (Abul-Fadl, M. A. M. & King, E. J.) 51
Phenylsulphuric acid, fate in rabbit (Garton, G. A. & Williams, R. T.) 158
Phenylsulphuric acid, rate of hydrolysis compared with that of phenylglucuronide (Garton, G. A., Robinson, D. & Williams, R. T.) 65
Phoca groenlandica (Greenland seals), vitamin A and liver fat of (Rodahl, K. & Davies, A. W.) 408
Phoca vitulina (common seals), vitamin A and liver fat of (Rodahl, K. & Davies, A. W.) 408
Phosphatase, intestinal, of rat, effect of diet on (Lawrie, W. R. & Yudkin, J.) 435
Phosphatase, serum alkaline, inactivation by adrenaline (Anderson, A. B.) xxxvi
Phosphate buffer, effect of concentration on succinic oxidase activity (Slater, E. C.) 8
Phosphate-buffered medium for use with kidney slices (Robinson, J. R.) 68
Phosphate, inorganic, formation from adenosinetriphosphate on storage (Bailey, K.) 479
Phosphate, reaction with mustard gas (Fleming, D. S., Moore, A. M. & Butler, G. C.) 546
Phosphatases, acid, of erythrocytes and of human prostate gland (Abul-Fadl, M. A. M. & King, E. J.) 51
Phosphates of calcium, solubility of (Robinson, J. R.) 68
Phosphatides in wheat starch (Rooke, H. S., Lampitt, L. H. & Jackson, E. M.) 231
Phosphoglucomutase, coenzyme of (Stickland, L. H.) xxi
Phospholipin determination in degenerating cat nerves (Johnson, A. C., McNabb, A. R. & Rossiter, P. J.) 500
Phospholipin in rabbit liver (Ada, G. L.) 422
Phospholipin metabolism in rabbit-liver cytoplasm (Ada, G. L.) 422
Phospholipins in whale liver (Barua, R. K. & Morton, R. A.) 308
Phospholipins, liver, effect of dietary oleic and palmitic acids in the composition and turnover rates of (Campbell, I. G., Olley, J. Blewett, M.) 105
Phospholipins of rats on diets containing pure fatty acids (Campbell, I. G., Olley, J. & Blewett, M.) 105
Phospholipins, P-precursor of (Popják, G. & Muir, H. M.) xi
Phospholipins, transmission through placenta (Popják, G. & Beeckmans, M.-L.) x
Phosphorus, acid-soluble, in hen eggs during development of embryo (Ramsay, W. N. M.) ix
Phosphorus compounds in cell nucleus (Davidson, J. N. & McIndoe, W. M.) xvi
Phosphorus compounds of wheat starch (Rooke, H. S., Lampitt, L. H. & Jackson, E. M.) 231
Phosphorus, inorganic, in foetal fluids of sheep during gestation (McDougall, E. I.) 387
Phosphorus, radioactive, use in study of metabolism of rabbit-liver fractions (Ada, G. L.) 422
Phosphorylaminoethanol, in calf embryo muscle (Gordon, A. H.) 99
Phosphorylase, detection in barley (Porter, H. K.) xxxvii
Phosphorylases, plant, inhibition by β-amylase (Porter, H. K.) xxxvii
Photography, ultraviolet, and paper chromatography (Markham, R. & Smith, J. D.) 294
Photometer, photoelectric flame (Domingo, W. R. & Klyne, W.) 400
Photopic vision, relation to vitamin A and retinene (Ball, S. & Morton, R. A.) 298
Pigments, acid-soluble, of molluscan shells (Comfort, A.) 199, 204, 208
Pisum sativum, chemical changes in carbohydrates during growth of (Eastham, M.) xvii
Placenta, mono-amine oxidase activity of (Thompson, R. H. S. & Tickner, A.) 125
Placenta, transmission of phospholipins through (Popják, G. & Beeckmans, M.-L.) x
Plant extracts, oxidation of manganese by, in the presence of hydrogen peroxide (Kenten, R. H. & Mann, P. J. G.) 255
Plant growth, relationship between constitution and effect of 2-phenoxyethylamine derivatives (Jones, R. L., Metcalfe, T. P. & Sexton, W. A.) 143
Plant phosphorylases, inhibition by β-amylase (Porter, H. K.) xxxvii
Plants, aneurin-inactivating mechanism of (Roberts, H. E., Evans, W. T. R. & Evans, W. C.) xxx
Plasma protein regeneration in renal disease with hypoproteinaemia and oedema (Herbert, F. K.) xxii
Poisoning, bracken, of farm animals (Roberts, H. E., Evans, E. T. R. & Evans, W. C.) xxx
Polydisperse material in the Gouy diffusiometer (Ogston, A. G.) 189
Polypeptides, arrangement in insulin (Sanger, F.) 563
Polypeptides, deamination by X-rays (Dale, W. M., Davies, J. V. & Gilbert, C. W.) 93

Polypeptides in nisin (Berridge, N. J.) 486
Polysaccharide, bacterial, determination of (Dagley, S, & Dawes, E. A.) 331
Polysaccharide content of *Escherichia coli* (Dagley, S. & Dawes, E. A.) 331; xxvi
Polysaccharides from *Penicillium luteum*, sedimentation and diffusion of (Ogston, A. G.) 189
Polysaccharides, new, isolation from *Penicillium luteum* (Freeman, G. G. & Macpherson, C. S.) 179
Polyuronic acid in *Byssochlamys fulva* (Beaven, G. H. & Brown, F.) 221
Porphyria, congenital, stercobilin formation in (Gray, C. H., Neuberger, A. & Sneath, P. H. A.) xvi
Porphyrin derivatives of *Corynebacterium diphtheriae* (Rawlinson, W. A. & Hale, J. H.) 247
Porphyrin metabolism in rabbit (Niven, J. S. F. & Neuberger, A.) iv
Porphyrins, biogenesis of (Muir, H. M. & Neuberger, A.) 163
Porphyrins in rabbits, biogenesis of (Muir, H. M. & Neuberger, A.) xxxiv
Porphyrins, shell, identification (Nicholas, R. E. H. & Comfort, A.) 220
Potassium in blood and urine, determination by flame photometer (Domingo, W. R. & Klyne, W.) 400
Potassium in foetal fluids of sheep during gestation (McDougall, E. I.) 397
Potassium ions, radioactive, transport through gastric mucosa (Crane, E. E. & Davies, R. E.) xxiii
Potato apyrase, use for assay of adenosinetriphosphate (Bailey, K.) 479
Potato, distribution and nutritive value of the nitrogenous substances in (Chick, H. & Slack, E. B.) 211
Potato eelworm hatching factor (Calam, C. T., Raistrick, H. & Todd, A. R.) 513; (Calam, C. T., Todd, A. R. & Waring, W. S.) 520; (Marrian, D. H., Russell, P. B., Todd, A. R. & Waring, W. S.) 524; (Russell, P. B., Todd, A. R. & Waring, W. S.) 528, 530; (Marrian, D. H., Russell, P. B. & Todd, A. R.) 533
Potency calculator for use with logarithmic dose response curves (Basil, B.) xvii
Prawn, astaxanthin in (Goodwin, T. W. & Srisukh, S.) 268
Precursor of phospholipids (Popják, G. & Beeckmans, M.-L.) xi
Pregnancy, composition of allantoic and amniotic fluids during gestation from ewes fed during (McDougall, E. I.) 397
Pregnancy in the rat, respiration and acid production of mammary tissue during (Folley, S. J. & French, T. H.) 270
Pregnane-3α:17α:20-triol, urinary, configuration at C-20 of (Klyne, W.) viii
Proliferation, effect on glucuronide-synthesizing system in mouse liver (Karunairatnam, M. C., Kerr, L. M. H. & Levvy, G. A.) 496
Propionamide and propionic acid, hydrolysis by rabbit-liver preparations (Bray, H. G., James, S. P., Thorpe, W. B. V., Wasdell, M. R. & Wood, P. B.) 467
Propionamide and propionic acid metabolism in rabbits (Bray, H. G., James, S. P., Thorpe, W. V., Wasdell, M. R. & Wood, P. B.) 467
Prostate gland, human, acid phosphatases of (Abul-Fadl, M. A. M. & King, E. J.) 51
Prosthetic groups of the cytochromes present in *Corynebacterium diphtheriae* (Rawlinson, W. A. & Hale, J. H.) 247
Protein and di-2-chloroethyl sulphide, antisera against complexes of (Fleming, D. S., Moore, A. M. & Butler, G. C.) 546
Protein hydrolysate, isolation of glucosamine and histidine from (Partridge, S. M.) 459
Protein in rabbit liver (Ada, G. L.) 422
Protein levels, dietary, influence on experimental liver injury in dogs (Bardawill, C. J. & Gornall, A. G.) iii
Protein nitrogen in foetal fluids of sheep during gestation (McDougall, E.) 397
Protein, plasma and tissue, regeneration in renal disease with hypoproteinaemia and oedema (Herbert, F. K.) xxii
Proteins, combination with suramin (Wilson, E. J. & Wormall, A.) 224
Proteins, hydrolysis of (Flowers, H. M. & Reith, (Reich), W. S.) xx
Proteins, in connective tissue, amino-acids of (Bowes, J. H. & Kenten, R. H.) 281
Proteins, isolation of L-threonine from (Elliott, D. F.) 429
Proteins of potato (Chick, H. & Slack, E. B.) 211
Proteus morganii, glutamic decarboxylase and glutaminase from (Hughes, D. E.) 325
Protocatechuic acid, fate in rabbit (Dodgson, K. S. & Williams, R. T.) 381
Protocatechuic aldehyde, fate in rabbit (Dodgson, K. S. & Williams, R. T.) 381
Protopectinase of *Byssochlamys fulva* (Beaven, G. H. & Brown, F.) 221
Psychotic patients, cholinesterase of serum and cerebrospinal fluid of (Early, D. F., Hemphill, R. E., Reiss, M. & Brummel, E.) 552
Purines in nucleic acids, identification and estimation of (Markham, R. & Smith, J. D.) 294
Pyrimidine bases in nucleic acid, identification and estimation of (Markham, R. & Smith, J. D.) 294
Pyrophosphate, formation of manganese complex by hydrogen peroxide and plant extracts (Kenten, R. H. & Mann, P. J. G.) 255
Pyrophosphate, inorganic, formation from adenosinetriphosphate on storage (Bailey, K.) 479
Pyruvate, fermentation by *Clostridium saccharobutyricum*, strain GR4 (Cohen-Bazire, G. & Cohen, G. N.) 41
Pyruvate-oxidase system in brain (Coxon, R. V., Liébecq, C. & Peters, R. A.) 320

Quaternary ammonium salts, herbicidal activity of (Jones, R. L., Metcalfe, T. P. & Sexton, W. A.) 143
Quaternary ammonium salts, respiration of rat-liver slices in presence of (Long, C.) xi
Quinine salt of eclepic acid (Calam, C. T., Todd, A. R. & Waring, W. S.) 520
Quotients, respiratory, manometric method for determination of (Laser, H. & Rothschild, Lord) 598

Rabbit, biogenesis of porphyrins in (Muir, H. M. & Neuberger, A.) xxxiv
Rabbit, biosynthesis of cyanophenylglucuronides by (Smith, J. N.) 638
Rabbit, fate of (+)-adrenaline, protocatechuic acid and protocatechuic aldehyde in (Dodgson, K. S. & Williams, R. T.) 381
Rabbit, fate of lower aliphatic amides in (Bray, H. G., James, S. P., Thorpe, V., W. Wasdell, M. R. & Wood, P. B.) 467
Rabbit, fates of phenol, phenylsulphuric acid and phenylglucuronide and metabolism of benzene (Garton, G. A. & Williams, R. T.) 158
Rabbit-liver cytoplasm, phospholipin metabolism in (Ada, G. L.) 422
Rabbit liver, distribution of glycogen in (Nys, A., Aubert, X. & Duve, C. de) 245
Rabbit, metabolism of toluic acids and amides (Bray, H. G., Thorpe, W. V. & Wood, P. B.) 45
Rabbit plasma, vitamin A following adrenalin injection (Goodwin, T. W. & Wilson, A. A.) 370
Rabbit, porphyrin metabolism in (Niven, J. S. F. & Neuberger, A.) iv

X-Radiation, deamination of nitrogenous compounds by (Dale, W. M., Davies, J. V. & Gilbert, C. W.) 93
α-Radiation from disintegration of boron, deamination of glycine by (Dale, W. M., Davies, J. V. & Gilbert, C. W.) 543
Radioactive iodine, absorption and excretion of DL-thyroxine labelled with (Clayton, J. C., Free, A. A., Page, J. E., Somers, C. F. & Woollett, E. A.) xx
Radioactive sodium and potassium ions, transport through gastric mucosa (Crane, E. E. & Davies, R. E.) xxiii
Rana temporaria, carotenoids, vitamin A and 7-dehydrosteroid in (Morton, R. A. & Rosen, D. G.) 612
Rape seed, germinating, activity of substituted β-phenoxyethylamines against (Jones, R. L., Metcalfe, T. P. & Sexton, W. A.) 143
Rat, conversion of acetate to glycine in (Arnstein, H. R. V. & Neuberger, A.) iii
Rat, effect of diet on intestinal phosphatase of (Lawrie, N. R. & Yudkin, J.) 438
Rat, irreversible deamination of threonine in (Elliott, D. F. & Neuberger, A.) xiii
Rat liver, amino-acid decarboxylases of (Sloane-Stanley, G. H.) 556
Rat-liver slices, respiration in presence of aliphatic amines, hydroxy-amines and quaternary ammonium salts (Long, C.) xi
Rat plasma, vitamin A level following adrenaline injection (Goodwin, T. W. & Wilson, A. A.) 370
Rat, respiration and acid production of mammary tissue during pregnancy, lactation and involution in (Folley, S. J. & French, T. H.) 270
Rats, adult and newborn, effects of glucose and calcium on the metabolism of kidney slices from (Robinson, J. R.) 68
Rats, degeneration in central nervous system and vitamin A deficiencies in (Coetzee, W. H. K.) 628
Rats, intestinal flora and nicotinamide metabolism (Ellinger, P. & Abdel Kader, M. M.) 276
Rats, nicotinamide methochloride excretion of (Ellinger, P. & Abdel Kader, M. M.) 276
Reductone, condensation products with sulphonamides (Bell, E. A., Cocker, W. & O'Meara, R. A. Q.) 373
Renal clearance of fructosans (Beattie, J. & Corcoran, A. C.) xv
Renal disease with hypoproteinaemia and oedema, plasma and tissue protein regeneration (Herbert, F. K.) xxii
Renal function, and unilateral kidney lesions, effect of cyanide (Nicholson, T. F.) 112
Reproduction and vitamin A in frog (Morton, R. A. & Rosen, D. G.) 612
Resins, ion-exchange, displacement chromatography on (Partridge, S. M.) 459
Resins, ion-exchange, fractionation of weak electrolyte mixtures on (Davies, C. W.) 38
Resistance, capillary, of guinea pigs (Zilva, S. S.) 79
Respiration of lactating mammary gland slices in presence of carbohydrates (Folley, S. J. & French, T. H.) 117
Respiration of mammary tissue during pregnancy, lactation and involution in the rat (Folley, S. J. & French, T. H.) 270
Respiration of rat-liver slices in the presence of aliphatic amines, hydroxyamines and quaternary ammonium salts (Long, C.) xi
Respiratory catalyst required for the reduction of cytochrome *c* by cytochrome *b* (Slater, E. C.) 14
Respiratory quotients, manometric method for determination of (Laser, H. & Rothschild, Lord) 598
Reticular tissue, amino-acid distribution of (Bowes, J. H. & Kenten, R. H.) 281
Reticular tissue, preparation of (Bowes, J. H. & Kenten, R. H.) 281
Retinae, sheep, metabolism of (Laser, H. & Rothschild, Lord) 598
Retinene, reactions with amino compounds (Ball, S., Collins, F. D., Dalvi, P. D. & Morton, R. A.) 304
Retinene, relation to photopic vision (Ball, S. & Morton, R. A.) 298
Rhodopsin-like substance from retinene (Ball, S., Collins, F. D., Dalvi, P. D. & Morton, R. A.) 304
Riboflavin deficiency interrelationship of vitamin B (Bhagvat, K. & Devi, P.) 32
Ribonucleic acids, quantitative analysis of (Markham, R. & Smith, J. D.) xxxii
Ribosides, purine and pyrimidine, chromatography of (Markham, R. & Smith, J. D.) 294
Ricinoleic acid and growth of *Corynebacterium* 'Q' (Pollock, M. R., Howard, G. A. & Boughton, B. W.) 417
Ring cleavage by means of a cell-free enzyme system (Parr, W. H., Evans, R. A. & Evans, W. C.) xxix
Root extracts, horse-radish, oxidation of manganese by (Kenten, R. H. & Mann, P. J. G.) 255
Roseins I, II and III from *Trichothecium roseum*, isolation and properties of (Freeman, G. G., Morrison, R. I. & Michael, S. E.) 191
Rotation, optical, of the molybdate complex of *dextro-iso*-citric acid (Eggleston, L. V. & Krebs, H. A.) 578

Salmon (*Salmo salar* L.), developing, distribution of ascorbic acid in (Daniel, R. J.) 435
Schistocerca gregaria Forsk, carotenoid distribution of (Goodwin, T. W.) 472
Schistocerca gregaria Forsk, carotenoids of the integument of (Goodwin, T. W. & Srisukh, S.) 263
Scurvy in guinea pigs, effect on capillary resistance (Zilva, S. S.) 79
Sea flea, astaxanthin in (Goodwin, T. W. & Srisukh, S.) 268
Seals, vitamin A in (Rodahl, K. & Davies, A. W.) 408
Sedimentation of polysaccharides from *Penicillium luteum* (Ogston, A. G.) 189
Seeds, germinating, activity of substituted β-phenoxyethylamines against (Jones, R. L., Metcalfe, T. P. & Sexton, W. A.) 143
Sericin, silk, isolation of serine from (Elliott, D. F.) 429
Serine, isolation from proteins (Elliott, D. F.) 429
Serine, separation from threonine (Elliott, D. F.) 429
Serum albumins, mobility in electrophoresis (Hoch, H.) 285
Serum alkaline phosphatase, inactivation by adrenaline (Anderson, A. B.) xxxvi
Serum cholinesterase of psychotic patients (Early, D. F., Hemphill, R. E., Reiss, M. & Brummel, E.) 552
Serum of newborn animals, cholinesterase activity of (McCance, R. A., Hutchinson, A. O., Dean, R. F. A. & Jones, P. E. H.) 493
Serum, sheep, and foetal fluids (McDougall, E. I.) 397
Shells, molluscan, acid-soluble pigments of (Comfort, A.) 199, 204, 208
Silica-gel columns, buffered, use in chromatographic separation of 2:4-dinitrophenyl amino-acids (Blackburn, S.) 579
Silicon, naturally occurring, in organic compounds (Holt, P. F. & Yates, D. M.) xxxii
Silk sericin, isolation of serine from (Elliott, D. F.) 429
Sodium in blood and urine, determination by flame photometer (Domingo, W. R. & Klyne, W.) 400
Sodium ions, radioactive, transport through gastric mucosa (Crane, E. E. & Davies, R. E.) xxiii
Sodium in foetal fluids of sheep during gestation (McDougall, E. I.) 397
Solanum nigrum as a source of the potato eelworm hatching factor (Russell, P. B., Todd, A. R. & Waring, W. S.) 528
Sphingomyelin determination in degenerating cat nerves (Johnson, A. C., McNabb, A. R. & Rossiter, R. J.) 500

Spore-bearing aerobes, germination of (Hills, G. M.) 353, 363
Squash (*Cucurbita pepo*), glutamic decarboxylase of (Hughes, D. E.) 325
Starch, wheat, phosphorus compounds of (Rooke, H. S., Lampitt, L. H., & Jackson E. M.) 231
Stearic acid and growth of *Corynebacterium* 'Q' (Pollock, M. R., Howard, G. A. & Boughton, B. W.) 417
Stercobilin formation in congenital porphyria and in the normal (Gray, C. H., Neuberger, A. & Sneath, P. H. A.) xvi
Stem of walnut, apparent vitamin C in (Wokes, F. & Melville, R.) 343
Steroid excretion, urinary, regulation of (Reiss, M., Hemphill, R. E., Gordon, J. J. & Cook, E. R.) 574
Steroids, 7-dehydro-, in the frog (Morton, R. A. & Rosen, D. G.) 612
Streptococcus agalactiae, inhibition by nisin (Berridge, N. J.) 486
Streptococcus cremoris, inhibition by nisin (Berridge, N.J.) 486
Streptococcus haemolyticus, breakdown of *N*-acetylglucosamine by strains of (Rogers, H. J.) 87
Subterranean organs of some Compositae, carbohydrates present in (Bacon, J. S. D. & Edelman, J.) xxviii
Succinate, aerobic oxidation of inhibitors of (Slater, E. C.) 8
Succinic dehydrogenase-cytochrome system in heart muscle and in kidney (Slater, E. C.) 1
Succinic oxidase system, effect of sulphydryl-combining compounds on activity of (Slater, E. C.) 130
Succinic oxidase system, inhibitors of (Slater, E. C.) 8
Succinic oxidase system, measurement of (Slater, E. C.) 1
Succinic oxidase system of heart-muscle and kidney preparations (Slater, E. C.) 14
Sucrose, dietary, effect on intestinal phosphatase of rat (Lawrie, N. R. & Yudkin, J.) 438
Sugar, effect on polysaccharide content of *Escherichia coli* (Dagley, S. & Dawes, E. A.) 331
Sugar formation in cress seedlings in relation to ascorbic acid synthesis (Mapson, L. W., Cruickshank, E. M. & Chen, Yu-Tuan) 171
Sugars, amino, metabolism of (Rogers, H. J.) 87
Sugars, determination of lactose in mixture (Malpress, F. H. & Morrison, A. B.) 455
Sugars in urine, paper-partition chromatography of (Eastham, M.) xiii
Sulphate, conjugation of xylene metabolites with (Bray, H. G., Humphris, B. G. & Thorpe, W. V.) 241
Sulphonamide derivatives, intracellular mode of action (Bell, E. A., Cocker, W. & O'Meara, R. A. Q.) 373
Sulphuric esters from rabbit urine after feeding 4-chlorocatechol and 4-chlororesorcinol (Dodgson, K. S. & Williams, R. T.) 381
Sulphydryl-combining compounds, effect on the activity of the succinic oxidase system (Slater, E. C.) 130
Suramin, combination with proteins (Wilson, E. J. & Wormall, A.) 224
Susceptibility, magnetic, of dihydroxyl haems (Keilin, J.) 448

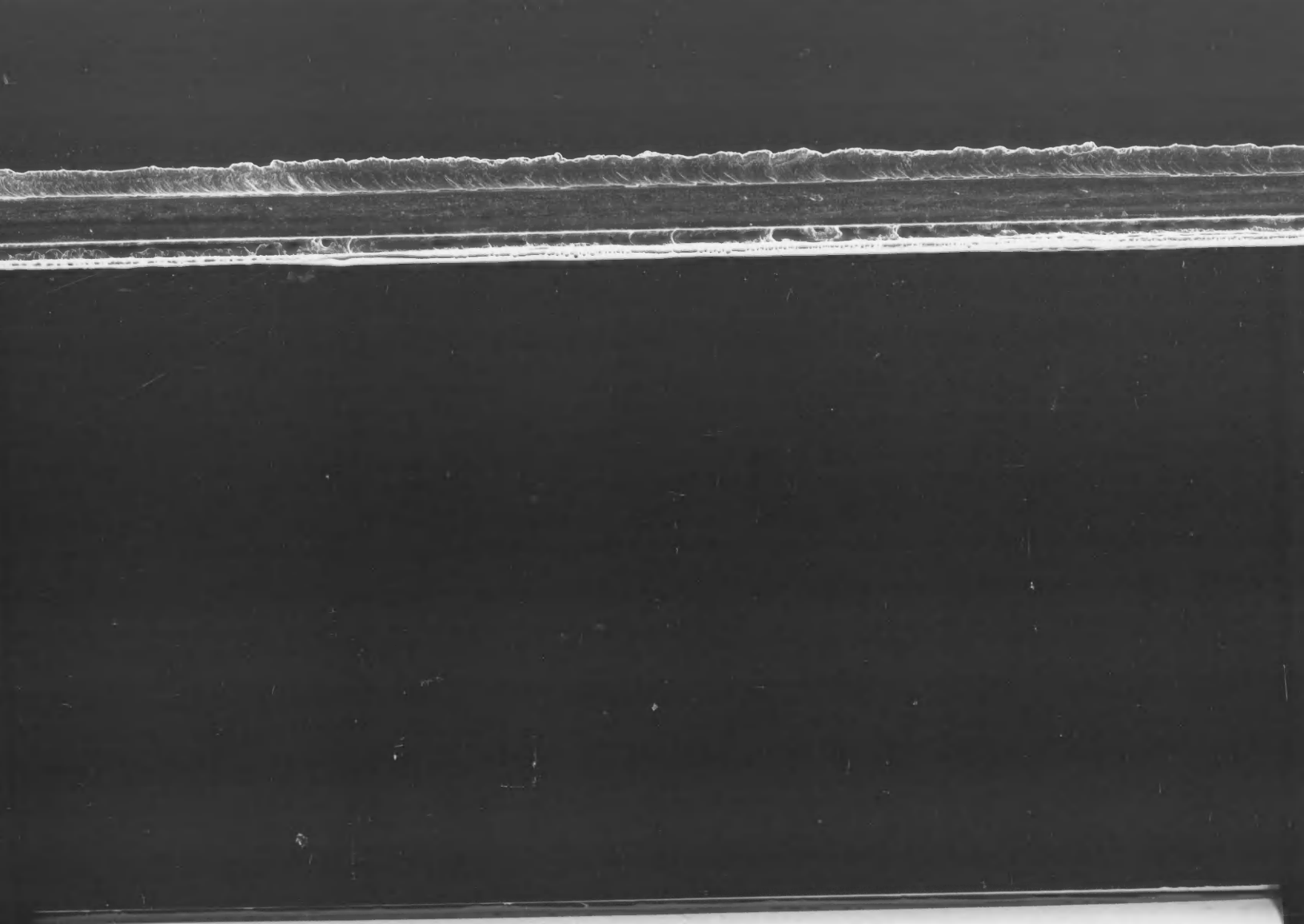
Tadpoles, occurrence of vitamin A and carotenoids in (Morton, R. A. & Rosen, D. G.) 612
D-Tartrate, effect on acid phosphatases (Abul-Fadl, M. A. M. & King, E. J.) 51
Tea manufacture, fermentation process in (Roberts, E. A. H.) 538
Tectibranch opisthobranchs, pigments of (Comfort, A.) 199
Terminal peptides of insulin (Sanger, F.) 563
Tetrapyrrole, possible nature of *Haliotis* pigments (Comfort, A.) 204
Tetrapyrroles, linear, occurrence in Turbinidae (Comfort, A.) 199
1:4-Thiazan-4-acetic acid from mustard gas and ethyl aminoacetate (Fleming, D. S., Moore, A. M. & Butler, G. C.) 546
Thiol groups and suramin (Wilson, E. J. & Wormall, A.) 224
Thiouracil, effect on carotene metabolism (Cama, H. R. & Goodwin, T. W.) 236
Thiouracil, effect on water-soluble vitamins in milk (Chanda, R., McNaught, M. L. & Owen, E. C.) xix
Threonine in rat, irreversibility of deamination of (Elliott, D. F. & Neuberger, A.) xiii
L-Threonine, isolation from proteins (Elliott, D. F.) 429
Thyroid, effect on β-carotene (Cama, H. R. & Goodwin, T. W.) 317
Thyroid, role in metabolism of carotene and vitamin A (Cama, H. R. & Goodwin, T. W.) 236
Thyroxine, effect on water-soluble vitamins in milk (Chanda, R., McNaught, M. L. & Owen, E. C.) xix
DL-Thyroxine labelled with radioactive iodine, absorption and excretion of (Clayton, J. C., Free, A. A., Page, J. E., Somers, G. F. & Woollett, E. A.) xx
Tigriopus fulvus Fisch., astaxanthin in (Goodwin, T. W. & Srisukh, S.) 268
Tissue protein regeneration in renal disease with hypoproteinaemia and oedema (Herbert, F. K.) xxii
Tissue, reticular, amino-acid distribution of (Bowes, J. H. & Kenten, R. H.) 281
Toluene derivatives, metabolism of (Bray, H. G., Humphris, B. G. & Thorpe, W. V.) 241
Toluic acids, fate in rabbit (Bray, H. G., Thorpe, W. V. & Wood, P. B.) 45
Tomato, roots, preparation of eelworm hatching factor from (Calam, C. T., Raistrick, A. R. & Todd, A. R.) 513
Transport of radioactive sodium and potassium ions through gastric mucosa (Crane, E. E. & Davies, R. E.) xxiii
Tricarboxylic acid cycle in brain (Coxon, R. V., Liébecq, C. & Peters, R. A.) 320
1:1:1-Trichloro-2:2-di(*p*-chlorophenyl)ethane (DDT), determination of chlorine in (Judah, J. D.) 60
Trichloroethylene, estimation in blood (Brain, F. H. & Helliwell, P. J.) 75
Trichothecin from *Trichothecium roseum* (Freeman, G. G., Morrison, R. I. & Michael, S. E.) 191
Trichothecium roseum Link, metabolic products of (Freeman, C. G., Morrison, R. I. & Michael, S. E.) 191
Trochidae, pigments of (Comfort, A.) 199
Tropomyosin, effect of electrolytes on molecular weight of (Adair, G. S., Bailey, K. & Tsao, T. C.) v
Trypanocides, combination with proteins (Wilson, E. J. & Wormall, A.) 224
Tryptophan, effect on nicotinamide metabolism (Ellinger, P. & Abdel Kader, M. M.) 276
Tryptophanase-tryptophan indole reaction, energy of activation of (Happold, F. C. & Morrison, G. A.) xx
Tuberin of potato (Chick, H. & Slack, E. B.) 211
Tubers, action of an enzyme preparation from *Helianthus tuberosus* L. (Jerusalem artichoke) on the carbohydrates present in (Edelman, J. & Bacon, J. S. D.) xxix
Turbinidae, pigments of (Comfort, A.) 199
Tyrosine, effect on germination of spores of *Bacillus anthracis* (Hills, G. M.) 363

Ultraviolet absorption of phenylglucuronidic acid (Garton, G. A., Robinson, D. & Williams, R. T.) 65
Ultraviolet photography and paper chromatography (Markham, R. & Smith, J. D.) 294
Umbilical cord blood, enzyme activities of (Jones, R. E. H. & McCance, R. A.) 464
Unsaturated fatty acids, long-chain, as essential bacterial growth factors (Pollock, M. R., Howard, G. A. & Boughton, B. W.) 417

Urinary steroid excretion, regulation of (Reiss, M., Hemphill, R. E., Gordon, J. J. & Cook, E. R.) 574
Urine albumins, mobility in electrophoresis (Hoch, H.) 285
Urine, determination of sodium and potassium in, by flame photometer (Domingo, W. R. & Klyne, W.) 400
Urine, foetal, of sheep (McDougall, E. I.) 397
Urine, human, determination of *N*-methyl-2-pyridone-3-(and -5-) carboxylamide (Holman, W. I. M. & Lange, D. J. de) 559
Urine, paper-partition chromatography of sugars in (Eastham, M.) xiii
Urine, pregnane-3α:17α:20-triol of, configuration at C–20 of (Klyne, W.) viii
Urohaem, dihydroxyl compounds of (Keilin, J.) 448
Uroporphyrin in molluscan shells (Nicholas, R. E. H. & Comfort, A.) 208
Uterus, mono-amine oxidase activity of (Thompson, R. H. S. & Tickner, A.) 125

n-Valeramide and valeric acid, hydrolysis by rabbit-liver preparation (Bray, H. G., James, S. P., Thorpe, W. V., Wasdell, M. R. & Wood, P. B.) 467
n-Valeramide and valeric acid metabolism in rabbits (Bray, H. G., James, S. P., Thorpe, W. V., Wasdell, M. R. & Wood, P. B.) 467
Vanadic acid, use in micro-estimation of citric acid (Weil-Malkerbe, H. & Bone, A. D.) 377
Vision, photopic, relation to vitamin A, and retinene, (Ball, S. & Morton, R. A.) 298
Vitamin A, absorption of (Eden, E. & Sellers, K. C.) xxxiii
Vitamin A, alleged formation from β-carotene treated with iodinated casein (Cama, H. R. & Goodwin, T. W.) 317
Vitamin A, alleged mobilization by adrenaline (Goodwin, T. W. & Wilson, A. A.) 370
Vitamin A, histological method for the biological estimation of (Coetzee, W. H. K.) 628
Vitamin A in seals (Rodahl, K. & Davies, A. W.) 408
Vitamin A in the frog, analytical methods for determination (Morton, R. A. & Rosen, D. G.) 612
Vitamin A in whale liver oil (Barua, R. K. & Morton, R. A.) 308
Vitamin A, locusts as possible source in native diets (Goodwin, T. W.) 472
Vitamin A, non-existence in locusts (Goodwin, T. W. & Srisukh, S.) 263
Vitamin A, preformed in northern krill (Kon, S. K. & Thompson, S. Y.) xxxi
Vitamin, A_1, relation to photopic vision (Ball, S. & Morton, R. A.) 298
Vitamin A, role of the thyroid in metabolism of (Cama, H. R. & Goodwin, T. W.) 236
Vitamin B_{12}, cup plate assay of (Cuthbertson, W. F. J. & Lloyd, J. T.) xvii
Vitamin B_{12} group of factors, paper chromatography of (Lester Smith, E. & Cuthbertson, W. F. J.) xii
Vitamin C, apparent, in walnut (Wokes, F. & Melville, R.) 343
Vitamin P. 3 (Zilva, S. S.) 79
Vitamins B in aneurin, riboflavin and biotin deficiencies (Bhagvat, K. & Devi, P.) 32
Vitamins, water-soluble, in milk, effect of thyroxine and thiouracil on (Chanda, R., McNaught, M. L. & Owen, E. C.) xix

Wallerian degeneration, lipid content of peripheral nerve during (Johnson, A. C., McNabb, A. R. & Rossiter, R. J.) 500
Walnut, apparent vitamin C in (Wokes, F. & Melville, R.) 343
Water, apparatus for micro-electrolysis of (Bentley, R.) 591
Water-soluble vitamins in milk, effect of thyroxine and thiouracil on (Chanda, R., McNaught, M. L. & Owen, E. C.) xix
Weak electrolyte mixtures, fractionation by ion-exchange resins (Davies, C. W.) 38
Weaning in the rat, respiration and acid production of mammary tissue during (Folley, S. J. & French, T. H.) 270
Whale liver oil analysis (Barua, R. K. & Morton, R. A.) 308
Wheat starch, phosphorus compounds of (Rooke, H. S., Lampitt, L. H., & Jackson E. M.) 231
Wings, locust, carotenoids of (Goodwin, T. W. & Srisukh, S.) 263

Xanthophyll in tadpoles (Morton, R. A. & Rosen, D. G.) 612
Xylenes, metabolism of (Bray, H. G., Humphris, B. G. & Thorpe, W. V.) 241

Yeast extract, effect on germination of *Bacillus anthracis* (Hills, G. M.) 353
Yeast, growth factors for *Corynebacterium diphtheriae* from (Chattaway, F. W., Dolby, D. E., Hall, D. A.& Happold, F. C.) 592
Yellow, indicator, and retinene (Ball, S., Collins, F. D., Dalvi, P. D. & Morton, R. A.) 304
Yields, ionic, in radiation processes, calculation processes (Dale, W. M., Davies, J. V. & Gilbert, C. W.) 93
Yolk of salmon egg, ascorbic acid in (Daniel, R. J.) 435